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A ^1H n.m.r. study of isotope exchange catalysed by glycolytic enzymes in the human erythrocyte

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The exchange of hydrogen and deuterium atoms between the C-2 position of lactate and solvent was monitored in suspensions of human erythrocytes by using a non-invasive spin-echo p.m.r. method that permits continuous assessment of the rate and the extent of exchange. Exchange rates were measured in cells suspended in buffers made in ^2H_2O and ^1H_2O after the addition of L-[2-^{1}H]lactate and L-[2-^{2}H]lactate respectively. The rate of exchange is dependent on the activities of four glycolytic enzymes (fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase and lactate dehydrogenase) and on the concentrations of their substrates. The dependence of the exchange on the following substrates was studied: (1) lactate, (2) the triose phosphates and fructose 1,6-bisphosphate and (3) pyruvate. Observation of the exchange *in vitro*, in a system produced by mixing the isolated enzymes, permits determination of the individual isotope-exchange equilibrium velocities of the enzymes. The dependence of the equilibrium velocity of human erythrocyte lactate dehydrogenase on $NAD^+ + NADH$ concentration was measured. Possible applications of these methods are discussed.

The non-invasive study of the properties of enzymes and the concentrations of metabolites in intact cells and tissues should lead to a better understanding of the control of metabolism and the relevance of enzyme properties observed *in vitro* to their properties *in situ*. We have shown (Simpson *et al.*, 1982*a*) that the properties of lactate dehydrogenase (EC 1.1.1.27) in human erythrocytes can be studied by monitoring the isotopic labelling of the lactate methyl group by p.m.r. As shown in the present paper, the p.m.r. method can also be used to study the exchange of the C-2 hydrogen atom of lactate with solvent. This exchange allows information about the kinetic properties of other glycolytic enzymes in the intact erythrocyte to be obtained.

The pathway for the exchange of hydrogen atoms between the C-2 position of lactate and solvent, as proposed by Rose & Warms (1969), is shown in Fig. 1. Four enzymes are involved: fructose bisphosphate aldolase (EC 4.1.2.13), triose phosphate isomerase (EC 5.3.1.1), glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) and lactate dehydrogenase. In the present paper we show how the overall isotope-exchange equilibrium velocities of these four enzymes can be measured in the intact erythrocyte and present a method for measuring the equilibrium velocities of the individual enzymes *in vitro*. Problems arising from possible kinetic isotope effects and the effect of endogenous lactate production on the measurement of the exchange in the intact cell are considered. The dependence of the overall equilibrium velocity in the cell on the lactate, pyruvate and triose phosphate concentrations is studied and discussed with respect to the individual isotope-exchange equilibrium velocities of the enzymes involved. A method is proposed for obtaining the glyceraldehyde phosphate dehydrogenase equilibrium velocity in the intact cell. With measurements of its equilibrium velocity *in vitro* this would allow a comparison of the properties of this enzyme *in situ* and *in vitro*. This is of interest, since the enzyme has been shown, under conditions of low ionic strength, to bind to the erythrocyte membrane with a consequent effect upon its activity (Eby & Kirtley, 1979). A recent study has suggested that two-thirds of the cellular glyceraldehyde phosphate dehydrogenase is membrane-bound in the intact cell (Kliman & Steck, 1980). The possible physiological significance, however, of this membrane association has yet to be determined.

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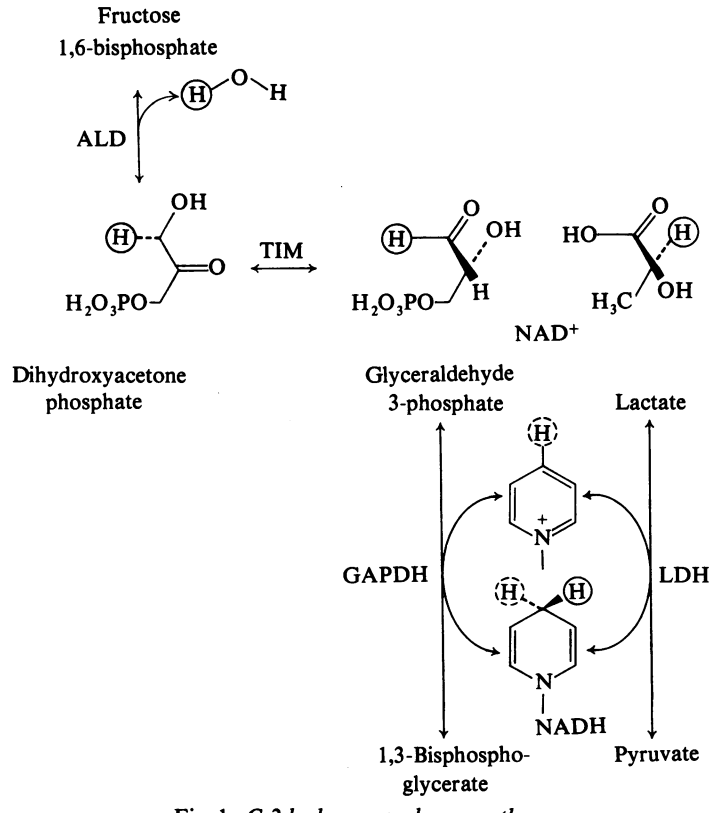

Fig. 1. *C-2 hydrogen-exchange pathway*

The pathway taken by the label exchanging between the C-2 position of lactate and solvent is shown. The exchanging isotope is ringed. Solvent isotope is stereospecifically incorporated into dihydroxyacetone phosphate by fructose bisphosphate aldolase (Rose & Warms, 1969). Abbreviations for the enzymes: LDH, lactate dehydrogenase; GAPDH, glyceraldehyde phosphate dehydrogenase; TIM, triose phosphate isomerase; ALD, fructose bisphosphate aldolase.

Experimental

Materials

Biochemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.), except for NAD^+, which was obtained from Boehringer Corp. (Lewes, East Sussex, U.K.). NADH was obtained in pre-weighed vials. Other chemicals were analytical grade. Fructose bisphosphate aldolase, glyceraldehyde phosphate dehydrogenase and triose phosphate isomerase, all from rabbit muscle, lactate dehydrogenase from human erythrocytes and lipoamide dehydrogenase from pig heart were obtained from Sigma. In ^2H_2O solutions, pH*, the uncorrected pH-meter reading, is quoted.

Preparation of L-[2-^{2}H]*lactate*

L-[2-^{2}H]Lactate was prepared by incubating lactate with lactate dehydrogenase, lipoamide dehydrogenase (EC 1.6.4.3) and NAD^+ as described by Alizade *et al.* (1975). The deuterated lactate was isolated by chromatography on an ion-exchange resin (Bartlett, 1959).

General methods

Human erythrocytes were prepared from 1-day-old blood (stored in citrate/phosphate/glucose) or from freshly drawn venous blood as described by Simpson *et al.* (1981). Erythrocyte lysates were prepared by freezing the cells in liquid N_2. Nicotinamide (10mM) was added to the cells before lysis in order to inhibit the NAD^+ nucleosidase (EC 3.2.2.5) present on the external surface of the erythrocyte (Alivisatos & Denstedt, 1951).

Determinations of enzyme activities and the concentrations of erythrocyte metabolites were made as described by Beutler (1975), except that all

enzyme assays were performed at pH7.4. Enzyme activities are expressed in terms of units (μmol of substrate consumed or product produced/min). All assays were performed at 37°C. Cell densities were determined by the micro haematocrit method (Hawksley micro haematocrit centrifuge).

Rabbit muscle fructose bisphosphate aldolase, glyceraldehyde phosphate dehydrogenase and triose phosphate isomerase, obtained as suspensions in $(NH_4)_2SO_4$ solution, were dialysed against 0.1% EDTA/1 mM-2-mercaptoethanol before use. Human erythrocyte lactate dehydrogenase was used as a suspension in $(NH_4)_2SO_4$ solution. In mixtures of the enzymes where the concentration of lactate dehydrogenase was varied, the concentration of $(NH_4)_2SO_4$ was also varied so that all the samples contained the same $(NH_4)_2SO_4$ concentration. [Experiments showed that $(NH_4)_2SO_4$, at the concentrations used, had no effect on the measured properties of the lactate dehydrogenase].

P.m.r. methods

Spectra were obtained at 470MHz by using a spin-echo sequence with $\tau = 68$ms (Brown *et al.*, 1977; Brindle *et al.*, 1979). Samples in ^1H_2O were run without a ^2H_2O lock, since field drift during accumulation of a spectrum was negligible. Spectra (112 scans each, collected in 2min) were stored on disc by a computer-controlled automatic data-acquisition routine, which also provided accurate timing for determination of exchange rates. Samples were run in 5mm-diameter tubes containing 0.5ml of solution or cell suspension. All samples were run at 37°C and preheated to this temperature before mixing. The pH values quoted were recorded at this temperature. All additions to the samples were of sufficiently small volume to have a negligible dilution effect. Concentrations in cell suspensions were calculated assuming that 72% of the cell volume is solvent water (Eilam & Stein, 1974).

Theory

Equilibrium velocity

The equilibrium velocity for the exchange of label between two pools, of concentrations A and B, may be calculated from the fractional progress towards equilibrium, f_e, by using the following equation (Boyer, 1959):

$$v = -2.3\frac{A \cdot B}{A+B} \cdot \frac{1}{\text{time}} \cdot \log(1-f_e) \qquad (1)$$

For C-2 hydrogen exchange, where one of these pools is solvent and is thus much larger than the lactate pool, the equilibrium velocity v can be calculated by multiplying the first-order rate constant for the exchange by the lactate concentration.

Relationship of the observed equilibrium velocity to the equilibrium velocities of the individual enzyme-catalysed steps in C-2 hydrogen exchange

For trace isotope exchange, the exchange rates of steps in series add reciprocally (Yagil & Hoberman, 1969). We have shown that under certain conditions this also applies to bulk isotope exchange. In the C-2 exchange system, if the concentration of lactate is significantly greater than that of the intermediate substrates involved in the exchange, the individual isotope-exchange equilibrium velocities of the enzymes can be related to the overall equilibrium velocity by the following equation (Foxall, 1981):

$$\frac{1}{V_{C2X}} = \frac{1}{V_{ALD}} + \frac{1}{V_{TIM}} + \frac{2}{V_{GAPDH}} + \frac{2}{V_{LDH}} \qquad (2)$$

where V_{C2X} is the measured overall equilibrium velocity for the system and V_{ALD}, V_{TIM}, V_{GAPDH} and V_{LDH} are the equilibrium velocities of fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase and lactate dehydrogenase respectively. The factors of 2 arise for the dehydrogenases because lactate dehydrogenase reacts stereospecifically with the A face of the coenzyme NADH, whereas glyceraldehyde phosphate dehydrogenase reacts stereospecifically with the B face. In order for the solvent isotope introduced on to the B face of the coenzyme by glyceraldehyde phosphate dehydrogenase to get to the C-2 position of the lactate molecule, it must pass twice through the reactions catalysed by glyceraldehyde phosphate dehydrogenase and lactate dehydrogenase.

Effects of enzyme activity and substrate concentration on the exchange rate

The interactions between metabolic flux, substrate concentrations and enzyme activities in multienzyme systems have been extensively discussed (see, e.g., Kacser & Burns, 1979). In the present paper we have used the Kacser & Burns (1979) definitions of Sensitivity and Elasticity in discussing the effect of enzyme and substrate concentrations on the rate of C-2 exchange. The Sensitivity coefficient, Z, is defined as:

$$Z = \frac{\delta F/F}{\delta E_t/E_t}$$

where F is metabolic flux and E is the catalytic activity of any one enzyme. Z describes the fractional change in flux due to a fractional change in enzyme activity. For the C-2 exchange system the value of an individual Sensitivity coefficient will depend on the equilibrium velocity of the enzyme in relation to others in the pathway. The equilibrium

velocity of an enzyme is dependent on its concentration and turnover number and on the concentrations of its substrates.

If a substrate concentration is changed in the C-2 exchange system, then the equilibrium velocity of one or more of the enzymes will also change. The fractional change in enzyme rate that occurs on a fractional change in substrate concentration has been designated the Elasticity coefficient (Kacser & Burns, 1979):

$$\frac{\delta v_i/v_i}{\delta S_j/S_j} = \varepsilon_{S_j}^{v_i}$$

where v_i is the enzyme rate for the enzyme E_i, S_j is substrate concentration and $\varepsilon_{S_j}^{v_i}$ is the Elasticity coefficient. For C-2 exchange this coefficient describes the fractional change in an enzyme's equilibrium velocity due to a fractional change in the concentration of one of its substrates.

Elasticity coefficients can also be applied to the effects of inhibitors and other factors in an enzyme's environment, such as pH and ionic strength, that will also affect the equilibrium velocity of the enzyme.

If a substrate concentration is varied in the multienzyme C-2 exchange system, then it will affect not just the equilibrium velocity of a single enzyme, but those of all those enzymes for which it is a common substrate or have a substrate with which it is in chemical equilibrium. However, by using the method shown in Fig. 4, the effect of substrate concentration on a single enzyme can be measured.

Isotope effects

Eqn. (2) was derived on the assumption that there are no kinetic isotope effects, i.e. that the equilibrium velocities of the enzymes are fixed for a given set of substrate concentrations and environmental conditions and are independent of the fractional labelling state of the substrates (Foxall, 1981). If there were significant kinetic isotope effects, then the equilibrium velocities would be time-dependent functions that change as the fractional labelling of the substrates change. Since the exchange is observed (*in vitro*) to follow simple first-order kinetics, this suggests that the kinetic isotope effects cannot be significant. The effects of a kinetic isotope effect can be minimized by making estimates of the rate constant from the half-time for the exchange or from the time course before this point.

Results and discussion

Observation of the exchange

When L-[U-^{1}H]lactate is added to erythrocytes in buffer made with ^2H_2O, the methyl-group resonance is initially inverted in the spin-echo spectrum (Fig. 2*a*) at this τ value (68ms) because of spin-coupling with the C-2 proton (Brown *et al.*, 1977; Campbell & Dobson, 1979). Deuterium substitution at the C-2 position results in heteronuclear coupling that produces a positive phase for the methyl-group resonance. Thus, as exchange at the C-2 position proceeds, the lactate methyl-group resonance changes from a negative to a positive peak, as shown in Fig. 2(*a*). When a C-2 deuteron is exchanged for a proton in buffer made with ^1H_2O (Fig. 2*b*), the sign of the phase change is reversed. The rate at which methyl-group peak inversion occurs gives the rate of exchange at the C-2 position. By observing inversion of the signal from the three protons of the methyl group, the effect of the exchange of the single proton or deuteron at the C-2 position is amplified. The C-2 proton can be observed directly, but this is difficult owing to its close proximity to the water resonance.

The exchanges shown in Figs. 2(*a*) and 2(*b*) *in vitro* display simple first-order kinetics with symmetrical peak inversion. Changes in the multiplicity of the methyl-group resonance (the inverted peak is a homonuclear coupled doublet and the upright peak a heteronuclear coupled triplet) have a negligible effect on the observed methyl-group peak height owing to the large linewidths in the observed spectra. Linewidths were further increased by an applied 16 Hz line broadening. Equilibrium velocities *in vitro* were calculated by multiplying the lactate concentration by a first-order rate constant obtained from a least-squares fit of the exchange time course.

In cells, because of endogenous lactate production, the time at which peak inversion occurs, referred to below as t_{null}, is used to calculate V_{C2X}, the overall equilibrium velocity. Endogenous lactate production during an exchange time course has a number of effects: (*a*) it dilutes the added lactate; (*b*) it affects the peak heights observed during the exchange time course of the added lactate; (*c*) it represents a source of lactate labelled with solvent isotope in addition to that produced by exchange.

At the point of peak inversion, the positive and negative components of the lactate methyl-group peak effectively cancel out and a null point is obtained. This point (t_{null}) represents the point at which 50% of all the lactate present is solvent labelled at the C-2 position. V_{C2X} can be calculated from t_{null} as follows.

If endogenous lactate production during an exchange time course is negligible and if L_0 and L_e are the concentrations of added and solvent-labelled lactate respectively at $t = 0$, then:

$$V_{C2X} = \frac{(L_0+L_e)}{t_{null}} \cdot \ln\left(\frac{2L_0}{L_0+L_e}\right) \qquad (3)$$

L_e is calculated by taking a spectrum before addition of the labelled lactate and from knowledge of the

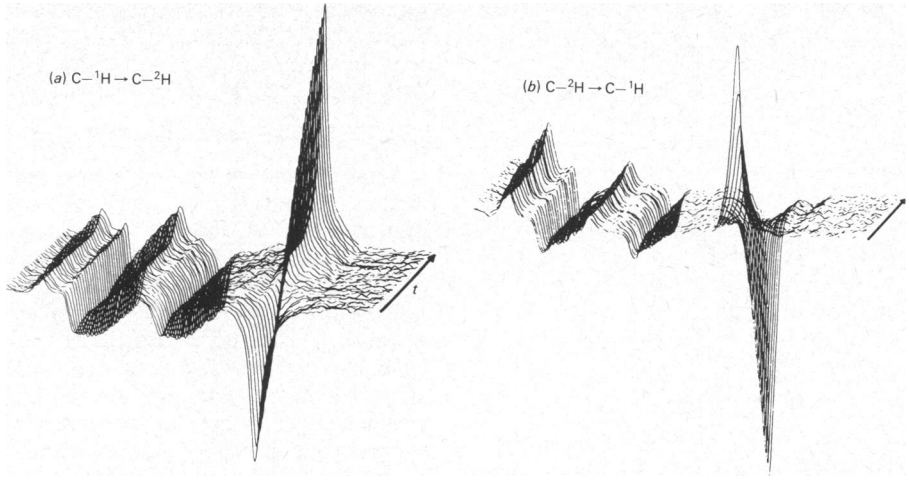

Fig. 2. *Stacked plots of lactate C-2 isotope-exchange time courses in erythrocyte suspensions in ^2H_2O and ^1H_2O*
(*a*) Inversion of the lactate methyl-group resonance after the addition of 12mM-L-[2-^{1}H]lactate to erythrocytes in Krebs–Ringer buffer made with ^2H_2O (75% haematocrit). Sequential 2min (112-scan) spin-echo ($\tau = 68$ms) spectra were accumulated after the addition of the lactate. (*b*) Inversion of the lactate methyl-group resonance after the addition of 12mM-L-[2-^{2}H]lactate to erythrocytes in Krebs–Ringer buffer made with ^1H_2O (75% haematocrit). Sequential 2min (112-scan) spin-echo ($\tau = 68$ms) spectra were accumulated after the addition of the lactate.

percentage labelling at the C-2 position of the added lactate.

If there is endogenous lactate production and the rate of this is small compared with V_{C2X}, then the following equation can be used to give an approximate correction for this effect:

$$V_{C2X} = k_1 \cdot \frac{\ln\left(\frac{2L_0}{Lt_{null}}\right)}{\ln\left(\frac{Lt_{null}}{L_0+L_e}\right)} \quad (4)$$

This equation was derived from an integrated rate equation that allowed for dilution of the lactate pool by endogenous lactate production and that assumed that solvent label was introduced into this pool at a constant rate, k_1, independent of that introduced by exchange.

Calculation of V_{C2X} from the point of peak inversion, where the fractional labelling state at the C-2 position is known, avoids the necessity of fitting the exchange time course to the integrated rate equation. This simplification would not be possible if the exchange were observed by monitoring the peak intensity of the C-2 proton directly. Furthermore the t_{null} method requires knowledge, not of the end point for the exchange, but only the degree of labelling of the lactate present at $t = 0$, which is readily obtained.

C-2 exchange in vitro

An exchange system *in vitro* was produced by mixing fructose bisphosphate aldolase, glyceraldehyde phosphate dehydrogenase and triose phosphate isomerase from rabbit muscle and human erythrocyte lactate dehydrogenase. NAD^+, fructose 1,6-bisphosphate and lactate were the only substrates added. The other substrates necessary for the exchange, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, NADH and pyruvate, are produced by equilibration of fructose 1,6-bisphosphate and lactate across the reactions catalysed by fructose bisphosphate aldolase and lactate dehydrogenase respectively. Exchange time courses were initiated by adding 12mM-L-[U-^{1}H]lactate to the enzyme mixture. The mixtures were preincubated with fructose 1,6-bisphosphate for approx. 5min before lactate addition in order to ensure that the fructose 1,6-bisphosphate had come to chemical and isotopic equilibrium. Both of these processes are expected to be rapid. Loss of protons from fructose 1,6-bisphosphate to solvent ^2H_2O, in the reaction catalysed by fructose bisphosphate aldolase, is considerably faster than the exchange of isotope at the lactate C-2 position. More important, however, is that the dihydroxyacetone phosphate and the other intermediate substrates involved in the exchange are present at much lower concentrations than is lactate. Relaxation of the fructose 1,6-bisphosphate–dihydroxyacetone phosphate–glyceraldehyde 3-phos-

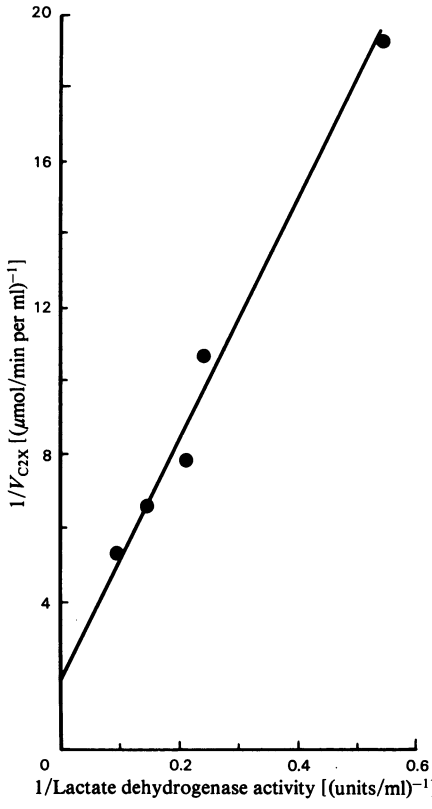

Fig. 3. *Plot of $1/V_{C2X}$ versus $1/A_{LDH}$ obtained in a C-2 exchange system in vitro*

The mixture of enzymes contained approx. 3 units of glyceraldehyde phosphate dehydrogenase/ml, 200 units of triose phosphate isomerase/ml and 0.3 unit of fructose bisphosphate aldolase/ml in 100mM-Tris/HCl buffer, pH* 7.4, 3mM-dithioerythritol and 0.5mM-EDTA. NAD^+ (100μM) and fructose 1,6-bisphosphate (200μM) were added approx. 5min before the start of a time course initiated by the addition of 12mM-L-[2-^{1}H]lactate. The exchange was monitored by collecting spectra (112 scans each) every 2min for 40min. Lactate dehydrogenase assays were performed at 37°C in 100mM-Tris/HCl buffer, pH 7.4, containing 0.5mM-EDTA. The oxidation of 0.2mM-NADH was monitored at 340nm after the addition of 1mM-pyruvate.

phate system to chemical equilibrium is rapid, since only 200μM-fructose 1,6-bisphosphate was added to mixtures containing approx. 0.3 unit of fructose bisphosphate aldolase (which has a very low K_m for fructose 1,6-bisphosphate; Mehler, 1963)/ml and approx. 200 units of triose phosphate isomerase/ml. Relaxation of the lactate–pyruvate system to chemical equilibrium is also rapid, attainment of equilibrium requiring an estimated conversion of 17μM-lactate and NAD^+ into pyruvate and NADH respectively. Although these experiments *in vitro* were performed in ^2H_2O, they can equally well be performed in ^1H_2O with L-[2-^{2}H]lactate.

Determination of an equilibrium velocity

Measurement of the exchange rate in enzyme mixtures, where the concentrations of three of the four enzymes involved in the exchange are held constant and that of the fourth is varied, allows the equilibrium velocity of the varied enzyme to be determined. The equilibrium velocity of human erythrocyte lactate dehydrogenase was determined by holding the concentrations of fructose bisphosphate aldolase, triose phosphate isomerase and glyceraldehyde phosphate dehydrogenase constant while the lactate dehydrogenase concentration was varied. Fig. 3 shows a plot of $1/V_{C2X}$ versus $1/A_{LDH}$, where A_{LDH} is lactate dehydrogenase activity measured spectrophotometrically. The linearity of the plot demonstrates that the C-2 exchange rate can be related to enzyme activity by the following equation:

$$\frac{1}{V_{C2X}} = \frac{1}{(V_{ATG})} + \frac{2}{\alpha A_{LDH}}$$

where the term $1/(V_{ATG})$ is the sum of the reciprocals of the equilibrium velocities for fructose bisphosphate aldolase, triose phosphate isomerase and glyceraldehyde phosphate dehydrogenase. The term $2/\alpha A_{LDH}$ is equivalent to $1/V_{LDH}$, the reciprocal of the lactate dehydrogenase equilibrium velocity. Thus the plot shown in Fig. 3 has a slope of $2/\alpha$ and an intercept on the ordinate of $1/V_{ATG}$. The term α is the specific equilibrium velocity of the lactate dehydrogenase. The value obtained for α, 0.064μmol of lactate exchanged/min per unit of enzyme activity, can be compared with a value of 0.057 calculated by using an equation derived by the method of Yagil & Hoberman (1969) for lactate dehydrogenase and using the kinetic constants of the bovine heart enzyme (Borgmann *et al.*, 1974) (see the Appendix for the equation). It should be pointed out, however, that the level of agreement may be coincidental, since the kinetic constants, in addition to being for the bovine heart enzyme, were obtained in ^1H_2O rather than ^2H_2O and in phosphate buffer, in which lactate dehydrogenase displays a higher affinity for NAD^+ and NADH than in Tris buffer (Winer & Schwert, 1958). Deuterium substitution also affects the binding of these coenzymes (Thomson *et al.*, 1964).

Effect of substrate concentration

The effect of the concentration of a substrate on an individual enzyme can be determined by measuring the specific equilibrium velocity of the enzyme at

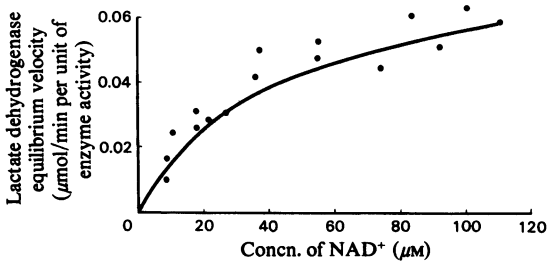

Fig. 4. *Dependence of the lactate dehydrogenase isotope-exchange equilibrium velocity on NAD$^+$ concentration in a C-2 exchange system in vitro*

The conditions of the experiment were similar to those described in the legend to Fig. 3. This experiment was repeated at various NAD$^+$ concentrations, and the lactate dehydrogenase equilibrium velocity was obtained at each NAD$^+$ concentration. The NAD$^+$ concentrations quoted are those of the NAD$^+$ added to the enzyme mixture. The slopes of the double-reciprocal plots, which were determined by logarithmic linear regression, were used to calculate lactate dehydrogenase equilibrium velocities.

a variety of concentrations of the particular substrate. For example, the dependence of the lactate dehydrogenase equilibrium velocity on NAD$^+$ concentration is shown in Fig. 4. The continuous line is a theoretical curve derived from the equation shown in the Appendix and the kinetic constants of the bovine heart enzyme.

Effect of an inhibitor

Fig. 5 shows the effect of oxamate, an inhibitor of lactate dehydrogenase (Novoa *et al.*, 1959), on V_{C2X} *in vitro*. The line drawn through the experimental points is a theoretical curve constructed with the assumption that oxamate is a simple competitive inhibitor of lactate dehydrogenase (see the Appendix). The other curves plotted in Fig. 5 show how the sensitivity of the exchange to inhibition of lactate dehydrogenase is altered by changing the Sensitivity coefficient of the enzyme. If the coefficient is decreased by increasing the lactate dehydrogenase concentration, then the exchange becomes less sensitive to inhibition of the enzyme. Fig. 5 demonstrates the relationship between Elasticity and Sensitivity with regard to the overall rate of exchange. A similar plot showing the effect of substrate concentration on the exchange is more complex, since it will change the equilibrium velocity of more than one enzyme.

The close fit of the experimental points to the theoretical curve demonstrates the precision of the n.m.r. technique for measuring the rate of isotope

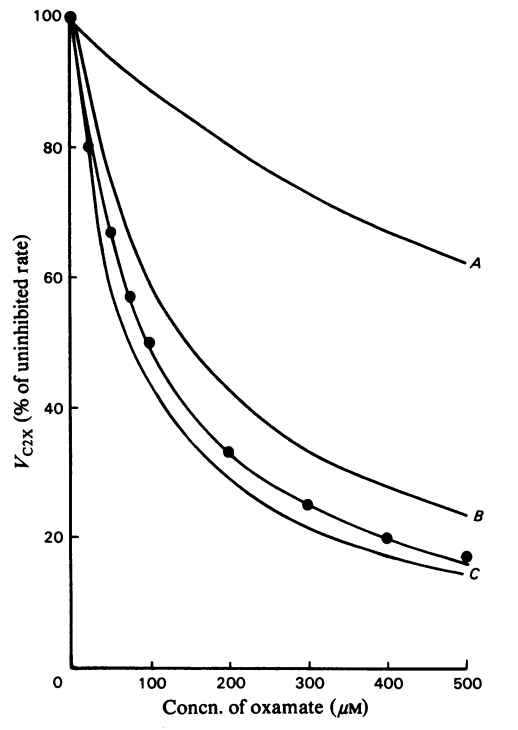

Fig. 5. *Effect of oxamate on the exchange rate observed in a C-2 exchange system in vitro*

The conditions of the experiment were similar to those described in the legend to Fig. 3. The line drawn through the experimental points is a theoretical curve constructed by assuming that there is simple competitive inhibition of lactate dehydrogenase by added sodium oxamate (see the Appendix). The other lines are theoretical curves showing the effect of changing the ratio of the lactate dehydrogenase equilibrium velocity to that of the other enzymes: curve *A*, $V_{LDH}/V_{ATG} = 10$; curve *B*, $V_{LDH}/V_{ATG} = 1.0$; curve *C*, $V_{LDH}/V_{ATG} = 0.1$.

exchange. In this experiment the only variable was the oxamate concentration.

This inhibition experiment can be performed with erythrocytes and used to estimate the Sensitivity coefficient of the enzyme *in situ*. A comparison of this coefficient, with that expected from the enzyme's activity in the cell and its known isotope-exchange properties *in vitro*, should yield information on the free concentrations of its substrates NAD$^+$ and NADH in the intact cell.

C-2 exchange in situ

In erythrocytes the glycolytic system is not at equilibrium but in a steady state. However, the enzymes involved in C-2 exchange, fructose bis-

phosphate aldolase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase and lactate dehydrogenase, are all near equilibrium in the erythrocyte (Minikami & Yoshikawa, 1966), i.e. the Mass-Action ratios of their substrates are similar to the equilibrium constants measured for these enzymes *in vitro*. It is this near-equilibrium condition that permits exchange of isotope. A further consideration in the intact cell is that of lactate transport across the cell membrane. This, however, is rapid at 37°C (Halestrap, 1976; Deuticke *et al.*, 1978; Brindle *et al.*, 1979), and thus V_{C2X} will be relatively insensitive to the rate of transport. This demonstrates a general point that in order to study the isotope-exchange properties of enzymes in intact cells a labelled molecule must be used that rapidly penetrates the cell membrane.

If the effect of substrate concentrations on the exchange *in situ* are to be examined, then these must be changed without destroying cellular integrity. For substrates transported across the cell membrane, e.g. lactate and pyruvate, this can be achieved by simple addition of these substrates to the cell suspension. For substrates to which the membrane is impermeable, then these must be changed indirectly, e.g. the triose phosphate concentrations can be raised by incubation at 4°C.

Effect of dihydroxyacetone phosphate concentration on the exchange

The relatively rapid rate of exchange at the lactate C-2 position, observed in intact cells, is dependent on the presence of high concentrations of dihydroxyacetone phosphate. For example, blood stored in citrate/phosphate/glucose shows no observable exchange and very low concentrations of the triose phosphates and fructose 1,6-bisphosphate (Bartlett & Barnet, 1960; Table 1). However, if erythrocytes are washed in Krebs–Ringer buffer at pH 7.4 and stored subsequently at 4°C for 24h or more, then the concentrations of the triose phosphates and fructose 1,6-bisphosphate can rise to very high values (Eckel *et al.*, 1966; Table 1). These cells catalyse rapid isotope exchange at the C-2 position of added lactate. Freshly drawn erythrocytes have very low concentrations of the triose phosphates. If the cells are cooled, however, the triose phosphate concentrations rapidly rise (Beutler, 1975), and lactate added to these cells again shows rapid isotope exchange at the C-2 position. Fig. 6 shows exchange time courses for L-[2-^{2}H]lactate incubated with intact cells and a lysate in ^1H_2O. The 12mM-lactate time course in the whole cells terminates before peak inversion is complete. This effect is even more marked in the case of the lysate. When 6mM-lactate was added to the same batch of cells, however, there was complete exchange. Dihydroxyacetone phosphate assays, details of which are given in the legend to Table 1, showed that before the addition of lactate to the intact cells there was 0.74 μmol of dihydroxyacetone phosphate/ml of cell water. This had fallen to 0.04 μmol/ml of cell water after incubation for 60min at 37°C. The decrease in dihydroxyacetone phosphate concentration in the lysate is faster, and a lysate incubated for 30min at 37°C showed no detectable dihydroxyacetone phosphate. These results imply that the premature termination of the exchange observed in Fig. 6 is due to a decrease in the concentrations of dihydroxyacetone phosphate and the other metabolites involved in the exchange with which it is in equilibrium, e.g. glyceraldehyde 3-phosphate. The peak heights shown in Fig. 6 are quoted as ratios of the peak height at $t = 0$ (obtained by extrapolation). Values in excess of −1.00 obtained for 6mM-lactate are due to endogenous lactate production and incomplete deuteration at the C-2 position of the added lactate. The simultaneous observation of

Table 1. *Comparison of triose phosphate and fructose 1,6-bisphosphate concentrations in erythrocytes stored in citrate/phosphate/glucose and in cells washed and stored for 24h at 4°C in Krebs–Ringer buffer, pH 7.4*

Concentrations were determined in neutralized perchloric acid extracts. Glyceraldehyde 3-phosphate was determined by measuring the increase in fluorescence as NAD^+ is reduced to NADH in the reaction catalysed by glyceraldehyde phosphate dehydrogenase. Arsenate was substituted for phosphate to make the reaction irreversible. Dihydroxyacetone phosphate was determined by adding triose phosphate isomerase, which converts dihydroxyacetone phosphate into glyceraldehyde 3-phosphate and leads to a further production of NADH. Fructose 1,6-bisphosphate concentration was determined by the addition of fructose bisphosphate aldolase, which cleaves fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Beutler, 1975).

	Concentrations (μmol/ml of cell water)		
Conditions of storage	Glyceraldehyde 3-phosphate	Dihydroxyacetone phosphate	Fructose 1,6-bisphosphate
Citrate/phosphate/glucose at 4°C	0	2	2
Krebs–Ringer buffer, pH 7.4, at 4°C	54	572	743

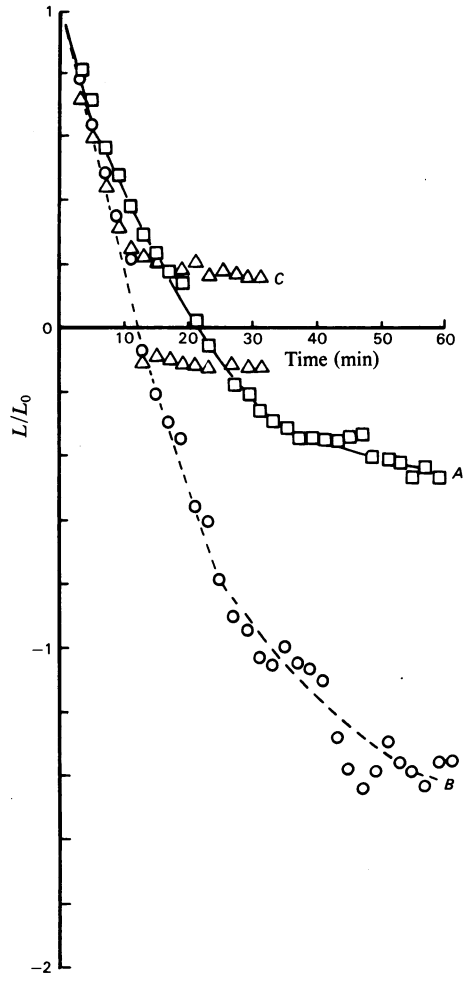

Fig. 6. *Effect of triose phosphate depletion on the exchange in erythrocytes*

Plots are shown of the ratio of peak height at time t (L) to peak height at $t=0$ (L_0) for L-[2-^{2}H]lactate incubated with intact erythrocytes and a lysate in Krebs–Ringer buffer made in ^1H_2O. Curve *A*, erythrocytes (76% haematocrit) with 12mM-lactate; curve *B*, erythrocytes (68% haematocrit) with 6mM-lactate; curve *C*, lysate prepared from erythrocytes with an haematocrit of 71%, with 12mM-lactate.

positive and negative methyl-group peaks at the point of peak inversion observed in the lysate case is due to the fact that deuterium substitution at the C-2 position results in a small upfield shift of the methyl protons.

The effect of changes in the triose phosphate concentrations on estimates of V_{C2X} are discussed in the next section.

Effect of lactate concentration on the exchange

The effect of lactate concentration on V_{C2X} is shown in Table 2. This shows V_{C2X} corrected for the effect of solvent-labelled lactate present at $t=0$ [corrected by using eqn. (3)] and corrected for both solvent-labelled lactate present at $t=0$ and for lactate production occurring between lactate addition at $t=0$ and the observed t_{null} [corrected by using eqn. (4)]. This latter correction is small, since the lactate production rate is small compared with V_{C2X}; in these cells a lactate production rate of approx. 4mM/h per ml of cells was observed. The correction for solvent-labelled lactate present at $t=0$ (L_e) can be minimized by thorough washing of the cells before lactate addition and by using lactate with a high degree of labelling at the C-2 position.

These experiments were performed in ^1H_2O. In ^2H_2O they are complicated by deuteration of the lactate methyl group (see, e.g., Simpson *et al.*, 1982*a*,*b*).

The observed t_{null} for the exchange, and thus the calculated V_{C2X}, are a function of the equilibrium velocities of all four enzymes involved and their substrate concentrations. If there are changes in the concentrations of these substrates during measurement of an equilibrium velocity, and, if these significantly affect the individual equilibrium velocities of the enzymes, then the observed t_{null} and the calculated overall equilibrium velocity will be affected. The magnitude of these substrate-concentration effects will depend on the individual Sensitivity and Elasticity coefficients of the enzymes.

We have already shown that the exchange depends on relatively high concentrations of the triose phosphates and that these decline at 37°C. The results in Table 2 show, however, that, above a certain value, changes in the concentrations of the triose phosphates, which are represented here by the dihydroxyacetone phosphate concentration, have a negligible effect on the calculated overall equilibrium velocity. In part A of Table 2 the dihydroxyacetone phosphate concentration decreases by approx. 90% between the observed t_{null} at low lactate concentration and that observed at high lactate concentration, and yet there is no significant difference in the calculated equilibrium velocity. In part B the dihydroxyacetone phosphate concentration decreases by only 20% between the t_{null} observed at high and low lactate concentrations respectively, and again there is no decrease in the calculated equilibrium velocity, and in fact there is an increase. The results in parts A and B also show that there is no significant dependence of the overall equilibrium velocity on lactate concentration, particularly at higher lactate concentrations. The lower values for V_{C2X} observed at lower added lactate concentrations in part B of Table 2 may be due to a lower equilibrium velocity of, for example, lactate

Table 2. *Effect of* L-[2-^2H]*lactate concentration on the overall equilibrium velocity in erythrocytes in Krebs–Ringer buffer made with* ^2H_2O

Equilibrium velocities were measured in packed-erythrocyte suspensions (84% haematocrit for the erythrocytes used in part A of the Table and 79% for part B). The quoted lactate concentrations were those added to the samples and were corrected for the excluded volume of the samples by assuming that 72% of the intracellular volume is solvent water (Eilam & Stein, 1974). The added lactate was 98% deuterated at the C-2 position as judged by proton n.m.r. The cells were washed just before use in order to lower the endogenous lactate concentration. This was approximately the same in all samples, at about 1 mM. Equilibrium velocities were calculated from the observed t_{null} (see the text). Dihydroxyacetone phosphate assays were performed on a parallel incubation by the method described in the legend to Table 1.

	Concn. of added lactate (mM)	Observed t_{null} (min)	Concn. of dihydroxyacetone phosphate at observed t_{null} (μmol/ml of cell water)	V_{C2X} corrected for solvent-labelled lactate at $t = 0$ (μmol/min per ml of cells)	V_{C2X} corrected for solvent-labelled lactate at $t = 0$ and for lactate production (μmol/min per ml of cells)
A	4.6	5	0.22	0.448	0.401
	6.9	10	0.16	0.398	0.355
	9.2	10	0.16	0.507	0.463
	11.5	12	0.14	0.545	0.502
	13.8	17	0.11	0.484	0.443
	16.1	18	0.10	0.529	0.487
	18.4	23	0.07	0.486	0.445
	23.0	31	0.03	0.457	0.417
B	4.5	7	1.08	0.357	0.288
	4.5	6	1.09	0.375	0.304
	9.0	12	1.04	0.469	0.406
	9.0	12	1.04	0.455	0.389
	13.6	18	0.98	0.495	0.434
	13.6	17	0.99	0.505	0.442
	18.1	23	0.94	0.520	0.459
	18.1	22	0.94	0.522	0.439
	22.6	27	0.90	0.551	0.491

dehydrogenase. They may also be due to the fact that the lactate concentration is no longer significantly greater than the concentrations of the intermediates involved in the exchange, in particular the triose phosphate and fructose 1,6-bisphosphate concentrations.

Since there is no significant dependence of V_{C2X} on lactate concentration (at relatively high concentrations of lactate) and on the concentrations of the triose phosphates (over the concentration ranges shown), then V_{C2X} will remain effectively constant during an exchange time course. The effect of lactate production may be regarded therefore as simply one of dilution of the added lactate, and this can be corrected for by using eqn. (4). The results in Table 2 show that this is a small correction. Insensitivity of V_{C2X} to the concentrations of lactate and the triose phosphates indicate that the Elasticity coefficients of the enzymes for these substrates are small over the concentration ranges shown.

In order to compare, by using this technique, the properties of glyceraldehyde phosphate dehydrogenase *in situ* and *in vitro* (see below), it is essential that the substrate equilibrium that exists *in vitro* approximates the near-equilibrium steady state that exists *in situ*. At relatively high concentrations of lactate and the triose phosphates the percentage changes in their concentrations *in situ* during measurement of V_{C2X} are small. Furthermore the Elasticity coefficients of the enzymes for these substrates are small in this concentration range. The effects of concentration changes occurring before observation of a null point are further decreased if the time taken to obtain a null is shortened. For example, in ^1H_2O, the time taken to reach a null point can be diminished by decreasing the percentage deuteration of the added lactate.

Effect of pyruvate concentration on the exchange

Fig. 7 shows the effect of pyruvate addition on the exchange of L-[2-^2H]lactate added to an erythrocyte suspension in ^1H_2O: the rate of exchange is decreased at higher pyruvate concentrations.

Addition of pyruvate to these cells will have a number of effects, i.e.: (*a*) it will rapidly increase the [NAD^+]/[NADH] ratio; (*b*) it will accelerate the long-term depletion of the triose phosphates (Rose & Warms, 1966); (*c*) it will lead to a transient increase in the 1,3-bisphosphoglycerate concentration (Rose & Warms, 1970). Each of these events can be

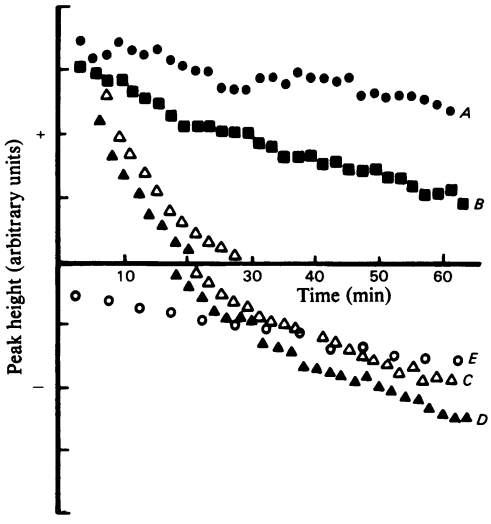

Fig. 7. *Inhibition by pyruvate of the exchange in erythrocytes*

Plots are shown of lactate methyl-group peak height versus time after the simultaneous addition to erythrocytes (70% haematocrit) of 12mM-lactate and (curve *A*) 5mM-pyruvate, (curve *B*) 1mM-pyruvate, (curve *C*) 0.5mM-pyruvate, (curve *D*) no pyruvate and (curve *E*) no addition of lactate or pyruvate.

expected to affect the equilibrium velocities of the enzymes involved in C-2 exchange, as outlined below.

An increase in the [NAD^+]/[NADH] ratio will affect the equilibrium velocities of both lactate dehydrogenase and glyceraldehyde phosphate dehydrogenase. An increase in the 1,3-bisphosphoglycerate concentration may inhibit glyceraldehyde phosphate dehydrogenase by formation of an enzyme–1,3-bisphosphoglycerate complex that is inactive in the exchange (the enzyme has a high affinity for 1,3-bisphosphoglycerate; Furfine & Velick, 1965). It should be noted that, if the currently accepted mechanism for the enzyme is correct (Fersht, 1977), then both P_i and 1,3-bisphosphoglycerate are not essential for the exchange of isotope between glyceraldehyde 3-phosphate and NAD^+ + NADH. This is supported by the results shown in the present paper for an exchange system *in vitro* that contained no added P_i and thus very little (if any) 1,3-bisphosphoglycerate. The observed inhibition of the exchange at high concentrations of pyruvate is not simply due to a total depletion of the triose phosphates. Dihydroxyacetone phosphate assays of the cells used in the experiment of Fig. 7 showed that before incubation at 37°C there was 0.71μmol of dihydroxyacetone phosphate/ml of cell water and that after incubation for 15min at 37°C in the presence of 5mM added pyruvate there was still 0.16μmol/ml of cell water present. At this added pyruvate concentration there was no detectable exchange (Fig. 7). In this case the decrease in intensity of the methyl-group peak of added lactate, which has a positive phase since the lactate is deuterated at the C-2 position, is due to an increase in endogenous lactate, which is protonated at the C-2 position and thus has an inverted methyl-group peak.

In addition to its indirect effects, pyruvate will also directly inhibit exchange of isotope across lactate dehydrogenase. This has been observed experimentally by Silverstein & Boyer (1964), and is predicted by an equation (shown in the Appendix) that relates the kinetics of the lactate dehydrogenase mechanism to its isotope-exchange equilibrium velocity. Pyruvate will also inhibit the exchange by the formation of abortive complexes with the enzyme (see, e.g., Simpson *et al.*, 1982*b*).

N.m.r. analysis of pyruvate inhibition of the exchange can only readily be demonstrated in ^1H_2O. In ^2H_2O deuteration of the pyruvate methyl group (Simpson *et al.*, 1981) leads to loss of lactate methyl-group peak intensity.

Conclusions

Isotope exchange at the lactate C-2 position has been studied previously by using radiolabelled L-[2-^{3}H]lactate in the erythrocyte (Rose & Warms, 1969) and in the liver (Hoberman, 1965; Bücher, 1969). The n.m.r. technique described in the present paper has permitted a much more detailed study of the exchange in the erythrocyte, since it is both non-invasive and provides rapid and continuous assessment of the isotopic composition of the exchanging species. The repeated sampling, extractions and separations required by radioactive-labelling techniques are thus eliminated, and the errors and labour involved are consequently diminished.

A disadvantage of the n.m.r. technique is its inherent lack of sensitivity. With the instruments used in the present work a reasonable threshold of detection in intact erythrocytes is approx. 0.5mM for the methyl group of lactate in a sample volume of approx. 0.5ml and a sampling time of 2min. However, it has been demonstrated that isotope exchange at the C-2 position of lactate can be employed to give, in an indirect manner, some assessment of isotope exchange in metabolites present in micromolar concentrations (e.g. NAD^+ + NADH and glyceraldehyde 3-phosphate).

It has been shown that in a mixture of the enzymes *in vitro* it is possible to determine the equilibrium velocity of a single enzyme by measur-

ing V_{C2X} at different concentrations of that enzyme. By using a similar approach we have made estimates of the glyceraldehyde phosphate dehydrogenase equilibrium velocity in the intact cell. If an enzyme can be specifically and irreversibly inhibited by a membrane-penetrating reagent, then its active concentration in the cell can be varied. By measuring V_{C2X} at different activities of the enzyme, the specific equilibrium velocity of the enzyme is obtained. Glyceraldehyde phosphate dehydrogenase can be irreversibly inhibited to various degrees in the intact erythrocyte by limited incubation with different concentrations of iodoacetate.

We consider that this technique could be used to study the properties of glyceraldehyde phosphate dehydrogenase, lactate dehydrogenase and possibly fructose bisphosphate aldolase and triose phosphate isomerase *in situ* and that comparisons could be made between their properties *in situ* and *in vitro*. Differences might be expected, e.g. if a significant amount of substrate is bound in the cell and not accessible to the enzyme. Recent work has indicated that the apparent lactate dehydrogenase activity observed in intact cells may be used to determine the cellular concentration of free $NAD^+ + NADH$ (Simpson *et al.*, 1982*b*).

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APPENDIX

Lactate dehydrogenase equilibrium velocity

The reaction mechanism for lactate dehydrogenase can be represented as follows (Borgmann *et al.*, 1974):

$$E+O \underset{k_{-1}}{\overset{k_{+1}[O]}{\rightleftharpoons}} EO \underset{k_{-2}}{\overset{k_{+2}[L]}{\rightleftharpoons}} EOL \rightleftharpoons ERP \underset{k_{-3}[P]}{\overset{k_{+3}}{\rightleftharpoons}} ER \underset{k_{-4}[R]}{\overset{k_{+4}}{\rightleftharpoons}} E+R$$

where O and L represent NAD^+ and lactate, and R and P represent NADH and pyruvate. It is assumed that EOL and ERP are rapidly interconverted.

The equilibrium velocity of an enzymic reaction can be related to the kinetic parameters of the reaction, as shown by Yagil & Hoberman (1969). These workers derived the following relationship for an equilibrium velocity of a reaction proceeding by n consecutive steps:

$$1/V_e = \sum_1^n 1/V_i$$

Thus the equilibrium velocity of lactate dehydrogenase for the exchange of isotope between the C-2 position of lactate and NADH can be related to the kinetic parameters of the enzyme by the following equation:

$$1/V_e = 1/[E]_t\left(\frac{1}{k_{-4}[R]}+\frac{k_{+4}}{k_{-3}k_{-4}[R][P]}+\frac{k_{+3}k_{+4}}{k_{-2}k_{-3}k_{-4}[R][P]}\right)\left(1+\frac{k_{-4}[R]}{k_{+4}}+\frac{k_{-3}k_{-2}k_{-4}[R][P]}{k_{+3}k_{+2}k_{+4}[L]}+\frac{k_{-3}k_{-4}[R][P]}{k_{+3}k_{+4}}\right)$$

where $[E]_t$ is the total enzyme concentration. This was calculated by dividing the measured activity for lactate dehydrogenase (assayed spectrophotometrically) by k_{-1}. This and the other kinetic constants used here were calculated for the bovine heart enzyme at 37°C and are from the data of Borgmann *et al.* (1974).

NAD-dependence

The line shown in Fig. 4 of the main paper was calculated by using the equation shown above. The lactate, pyruvate, NAD^+ and NADH concentrations were estimated by using an equilibrium constant of 1.11×10^{-11} (Williamson *et al.*, 1967). Fluorimetric measurements of variation in the NADH concentration in the enzyme mixture with various lactate and pyruvate concentrations (results not shown) confirmed that the NAD^+ and NADH concentrations were determined by the lactate dehydrogenase equilibrium.

Oxamate inhibition (fit to Fig. 5 of main paper)

Kinetic and binding studies (Novoa *et al.*, 1959*a*; Novoa & Schwert, 1961) have shown that oxamate binds to both the NAD^+-bound and NADH-bound forms of lactate dehydrogenase, although it has a higher affinity for the latter form.

The dissociation constant for NADH on bovine heart lactate dehydrogenase has been estimated to be 2.5×10^{-7} M in Tris buffer, pH 7.08 and I 0.2 (Novoa *et al.*, 1959*b*). The dissociation constant for NAD^+ binding has been estimated to be 3.9×10^{-3} M in 0.1 M-sodium phosphate buffer, pH 6.8 (Takenaka & Schwert, 1956). The NAD^+ and NADH concentrations present in the experiment depicted in Fig. 5 of the main paper were estimated to be 83 and 17 μM respectively. Thus, under the conditions of this experiment and assuming that human erythrocyte lactate dehydrogenase has similar kinetic properties to the bovine heart enzyme, then the enzyme will be predominantly in the NADH-bound form. Oxamate inhibition of the exchange can thus be regarded as a case of simple competitive inhibition, where oxamate competes with pyruvate for binding to the NADH-bound form of the enzyme.

For competitive inhibition

$$v = \frac{VS}{S+K(1+I/K_i)}$$

where v is the rate of the enzyme-catalysed reaction, V is the rate of the reaction at infinite concentration of substrate, S is substrate concentration, I is inhibitor concentration, K_i is the dissociation constant for the enzyme–inhibitor complex and K is the dissociation constant for the substrate. By equating v with the isotope-exchange equilibrium velocity this equation was used to calculate the percentage decrease in the lactate dehydrogenase equilibrium velocity at various oxamate concentrations and with various values for K_i. A pyruvate concentration of 17 μM was used in the calculations, since this is the

concentration estimated to be present when 12mM-lactate is added to an enzyme mixture containing 100μM-NAD^+, which were the conditions of the experiment shown in Fig. 5 of the main paper. A value of 71μM was chosen for the dissociation constant of pyruvate by equating a reported K_m value, for human erythrocyte lactate dehydrogenase (Wang, 1977), with K. The effect of a percentage decrease in the lactate dehydrogenase equilibrium velocity on the overall exchange of isotope at the C-2 position of lactate can be calculated by using eqn. (2) (see the main paper). If oxamate inhibits only lactate dehydrogenase, then the effect of a percentage decrease in the lactate dehydrogenase equilibrium velocity can be expressed as a percentage decrease in V_{C2X}. The experimental points shown in Fig. 5 of the main paper were fitted by using a K_I value of 60μM, which is similar to that obtained for the bovine heart enzyme by steady-state kinetic analysis and from binding studies (Novoa *et al.*, 1959*b*; Novoa & Schwert, 1961).

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