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Studies of pyruvate–water isotope exchange catalysed by erythrocytes and proteins

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Erythrocyte suspensions in buffer made with ^2H_2O catalyse the exchange of pyruvate protons. This process can be easily observed by using spin-echo proton magnetic resonance. The dominant exchange process is shown to be due to the formation of Schiff-base links between pyruvate and amino groups of haemoglobin. Other proteins with free α-amino groups also catalyse the exchange. The pH*-dependence of the exchange rate due to hen-egg-white-lysozyme reflects the dissociation of the α-amino group.

Spin-echo p.m.r. techniques have recently been applied in studies of metabolism and transport in intact erythrocytes (Brown *et al.*, 1977; Brindle *et al.*, 1979). These studies revealed that the protons of pyruvate and lactate exchange with solvent water. It is important to understand such exchanges, not only because they affect the interpretation of resonance intensity in terms of concentration, but also because isotope exchange can be of great value in giving information about metabolism (Katz & Rognstad, 1976) and the mechanism of enzymes (Rose & Rose, 1969).

The exchange of the methyl hydrogen atoms of pyruvate with solvent water has been observed to be catalysed by several enzymes, including oxaloacetate decarboxylase (E.C. 4.1.1.3) (Kosicki, 1968), pyruvate kinase (E.C. 2.7.1.40) (Rose, 1960), glutamate–pyruvate transaminase (E.C. 2.6.1.2.) (Rognstad & Wals, 1976) and 2-keto-3-deoxy-6-phosphogluconate aldolase (E.C. 4.1.2.14) (Meloche *et al.*, 1977). This exchange has been proposed as a possible measure of pyruvate cycling around pyruvate kinase, pyruvate carboxylase (E.C. 6.4.1.1) and phosphoenolpyruvate carboxykinase (E.C. 4.1.1.32) (Rognstad, 1979).

We set out to identify the catalyst of the observed hydrogen exchange of pyruvate in erythrocytes with the hope that this could be used as a probe of erythrocyte biochemistry. As will be demonstrated in this paper, the principal catalysts are the amino groups of haemoglobin, which means that there is little scope for the application of pyruvate hydrogen exchange to studies of erythrocyte metabolism. There are possibilities, however, for using the exchange as a probe of the environment and ligand state of the α-amino groups of proteins.

Experimental

Materials

Proteins, enzymes and biochemicals were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.) except for carbonic anhydrase [from Seravac Laboratories (Maidenhead, Berks., U.K.)]. Other chemicals were analytical grade. Proteins were dissolved in ^2H_2O and the pH* (uncorrected pH meter reading) was adjusted with NaO^2H or ^2HCl. 2-Hydroxy-2-methyl-4-oxoglutarate was prepared by the method of De Jong (1901).

Preparation of erythrocytes

Human erythrocytes were prepared from blood bank cells, stored in citrate/phosphate/glucose, by washing once in 10 vol. of phosphate-buffered saline [5 mM-sodium phosphate (pH 7.4)/0.15 M-NaCl in water] and removing the 'buffy coat' by aspiration. The cells were then washed five times in 1 vol. of Krebs–Ringer buffer (Krebs & Henseleit, 1932) made in either H_2O or ^2H_2O as appropriate. Bovine blood from slaughtered animals was stored in citrate/phosphate/glucose and the erythrocytes, in Krebs–Ringer buffer made with ^2H_2O were prepared similarly. Horse blood was drawn into 10 vol. of phosphate-buffered saline and then the cells were washed into Krebs–Ringer buffer made with ^2H_2O. All washes were performed at room temperature.

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Preparation of human haemoglobin

Human erythrocytes were washed with phosphate-buffered saline and lysed by the addition of 20vol. of deionized water. The membranes were removed by centrifugation for 15min at 30000*g*. The haemoglobin was then precipitated twice by adding saturated $(NH_4)_2SO_4$, concentrated against solid $(NH_4)_2SO_4$ and dialysed against deionized water to remove the salt. This procedure yielded haemoglobin solutions of up to 2mM with no detectable organic phosphate [assayed by the method of Fiske & Subbarow (see Leloir & Cardini, 1957)]. Deoxyhaemoglobin was obtained by incubating haemoglobin solutions under humidified N_2 in a tonometer (Instrumentation Laboratory no. 237). Further purification of human haemoglobin was obtained by $(NH_4)_2SO_4$ fractionation followed by the application of the 30–70%-satd. fraction (dialysed against 0.1M-Tris/HCl, pH8.3) to a column (1cm × 20cm) of DEAE–Sephadex equilibrated wtih the same buffer; elution was with 0.1M-NaCl/0.1M-Tris/HCl, pH8.3. The fractions containing haemoglobin were pooled, precipitated with 70%-satd. $(NH_4)_2SO_4$, concentrated and dialysed as above. Haemoglobin in ^2H_2O was obtained by dialysing it five times against 1vol. of ^2H_2O.

Preparation of [$^2H_3$]pyruvate

[$^2H_3$]Pyruvate was prepared by incubating 1M-sodium pyruvate with 1mM-haemoglobin in ^2H_2O at 37°C for 6h. The solution was then deproteinized by heating for 10min at 95°C and centrifuging on an Eppendorf centrifuge (type 5412). The resulting pyruvate solution was assayed enzymically (Beutler, 1975) with NADH and lactate dehydrogenase (E.C. 1.1.1.28) on a Hilger–Watts H700 Uvispek).

General methods

Cell densities were determined with the microhaematocrit method (Hawksley microhaematocrit centrifuge). Haemoglobin concentrations were determined from the absorbance at 540nm of the cyanometderivative (Beutler, 1975). Pyruvate kinase was determined by using a coupled assay with lactate dehydrogenase (Beutler, 1975) and is expressed in terms of units (μmol of NADH oxidized/min).

Proton magnetic resonance methods

Erythrocyte spectra in ^2H_2O buffer at 270MHz were obtained by using a spin-echo sequence with $\tau = 60$ms as described previously (Brown *et al.*, 1977). Spectra in H_2O were obtained similarly at 470MHz. Exchange of pyruvate protons in ^2H_2O buffer (120mM-KCl/1mM-KH_2PO_4/2.5mM-$CaCl_2$/0.6mM-$MgSO_4$/0.6mM-$MgCl_2$/50mM-sodium pyruvate, pH*7.4) by rabbit muscle pyruvate kinase was followed using a Perkin–Elmer R12 60MHz n.m.r. spectrometer. Rate constants for exchange were determined by logarithmic linear regression of peak height, normalized to an internal peak (see Brindle *et al.*, 1979). Errors were derived from the standard deviation of the regression slope. The lysozome spectra used for detecting the chemical shift of the α-CH resonance of lysine-1 were normal Fourier transform convolution difference spectra (270MHz) of a 5mM solution of the protein in ^2H_2O (Campbell & Dobson, 1979). All experiments were performed at 37°C.

Results and discussion

When millimolar quantities of pyruvate are added to high haematocrit suspensions of human erythrocytes in Krebs–Ringer buffer made in ^2H_2O, the pyruvate proton peak is easily observed by using spin echo p.m.r. Also visible, especially at higher pyruvate concentrations, are two further peaks due to the addition of pyruvate. The chemical shifts of these peaks, 1.32 and 1.44p.p.m., correspond to those of the dimer (2-hydroxy-2-methyl-4-oxoglutarate) and the hydrate (2,2-dihydroxypropionate) (Pocker *et al.*, 1969). The intensity of these peaks relative to the pyruvate peak is not a direct measure of their relative concentrations in this spin-echo

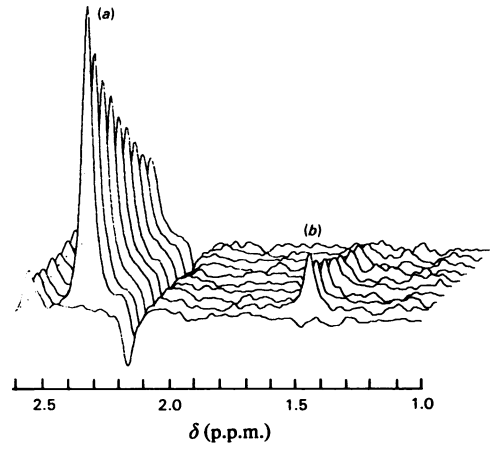

Fig. 1. *Stacked plot of pyruvate isotope-exchange time course in an erythrocyte suspension*

Sequential 3min (168 scan) spin-echo ($\tau = 60$ms) spectra accumulated before ($t = -1.5$min) and after addition of 20mM-pyruvate to 0.5ml of human erythrocytes in Krebs–Ringer buffer made in ^2H_2O (75% haematocrit). Chemical shifts are downfield from trimethylsilylpropanesulphonic acid. The peaks are (*a*) pyruvate and (*b*) pyruvate hydrate. The dimer peak (see the text) is not obvious at this pyruvate concentration due to the inverted haemoglobin peaks in that region.

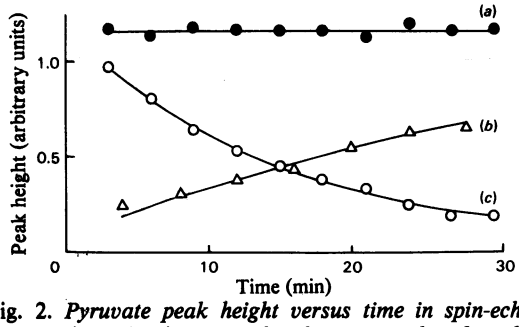

Fig. 2. *Pyruvate peak height versus time in spin-echo spectra ($\tau = 60$ ms) accumulated successively after the addition of 20 mM-pyruvate to (a) ^2H_2O, (b) packed erythrocytes in Krebs–Ringer buffer made in ^2H_2O and (c) packed erythrocytes in Krebs–Ringer buffer made in H_2O*

The pH* at the end of the experiment in (*a*) was approx. 7.5. In (*b*) the pyruvate was initially 95% deuterated. In (*b*) the haematocrit was 78% and in (*c*) it was 82%. The lines for (*b*) and (*c*) were obtained from a least-squares fit to the appropriate exponential function.

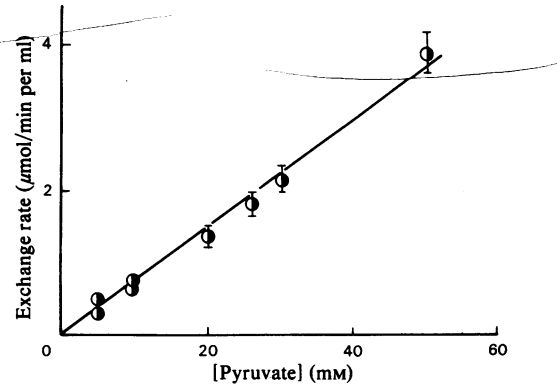

Fig. 3. *Variation of the initial rate of pyruvate exchange with pyruvate concentration in erythrocyte lysates (80% initial haematocrit).*

The vertical bars indicate the standard deviation of the points obtained from logarithmic linear regression of peak height versus time.

experiment; the true concentrations of these compounds in a sample containing 10 mM-pyruvate is less than 0.5 mM for the hydrate and less than 0.1 mM for the dimer (estimated from normal Fourier transform spectra in 10 mM-imidazole, pH*7.4) respectively.

If spectra are accumulated successively and the time-dependent behaviour of the peaks followed, the pyruvate peak is observed to decay exponentially (see Figs. 1 and 2). The hydrate peak also decays in parallel, as it is in equilibrium with the pyruvate (see Fig. 1); the dimer peak does not change. This signal loss could be due to binding of pyruvate to a macromolecule, metabolism of the pyruvate or solvent exchange of the protons. Since pyruvate transport in human erythrocytes at 37°C is extremely rapid (Halestrap, 1976), the concentration of pyruvate in free solution in the whole sample may be determined by centrifuging and assaying the supernatant. When this is done, no significant loss of pyruvate is observed, implying that solvent exchange is the cause of the signal loss. In confirmation, when [$^2H_3$]pyruvate is added to packed cells in Krebs–Ringer buffer made with H_2O, a pyruvate methyl signal is observed to grow over a similar time course in the p.m.r. experiment (see Fig. 2). The linearity of the semi-logarithmic plots (correlation coefficient 0.95–0.995) implies that changes in the T_2 of the pyruvate protons as deuteration of the methyl group proceeds are insignificant. Further evidence for this conclusion is that, within experimental error, the observed decay constant is independent of the delay time 2τ.

Some of the enzymes that are known to catalyse pyruvate hydrogen–solvent exchange are present in erythrocytes at significant activities, namely pyruvate kinase and glutamate–pyruvate transaminase (Friedemann & Rapoport, 1974). Addition of 3 mM-cycloserine, an inhibitor of the glutamate–pyruvate transaminase-catalysed exchange (Rognstad & Wals, 1976) has no effect on the exchange rate in erythrocytes (see Table 1). The human erythrocyte has approx. 4 units of pyruvate kinase activity/ml of packed cells (Friedemann & Rapoport, 1974). When an equivalent activity of rabbit muscle pyruvate kinase is added to 50 mM-pyruvate at pH*7.4 in ^2H_2O buffer (see the Experimental section) the $t_{\frac{1}{2}}$ for the decay of the pyruvate peak in the p.m.r. spectrum is of the order of 60 h (see also Rose, 1960). Packed erythrocytes are capable of exchanging 50 mM-pyruvate with a $t_{\frac{1}{2}}$ of 10 min (see Fig. 3).

Packed erythrocyte samples may be lysed by repeated freezing and thawing prior to the pyruvate exchange-rate measurement. The same rate of exchange is observed with lysed cells as with whole erythrocytes. In such experiments, however, inhibitors and metabolites that do not permeate the cell membrane may be added and the effect studied. Addition of high concentrations ($100 \times K_i$) of the potent pyruvate kinase inhibitor phosphoglycollate (Nowak & Mildvan, 1970) to a lysate has no effect on the pyruvate exchange even in the presence of the added co-substrate ADP (see Table 1). The dependence of the exchange rate on pyruvate concentration (see Fig. 3), shows no sign of a saturation effect up to 50 mM-pyruvate. Bovine and horse erythrocytes exhibit exchange-rate constants very

similar to that of human cells [$2.1(\pm0.3)\times10^{-4}$, $2.0(\pm0.2)\times10^{-4}$ and $1.8(\pm0.2)\times10^{-4}$ per minute per mg of haemoglobin per ml respectively] despite considerable variance in the activities of many erythrocyte enzymes between these species (Friedemann & Rapoport, 1974). These results imply that a specific enzyme is not the primary catalyst of the exchange.

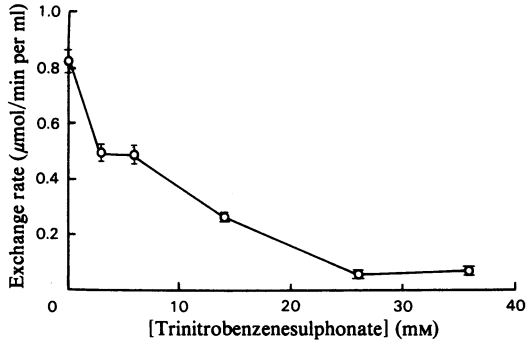

Fig. 4. *Progressive inhibition of pyruvate hydrogen exchange in erythrocyte lysates by trinitrobenzenesulphonate*

Samples of lysed packed erythrocytes (83% haematocrit) were incubated for 30 min with various concentrations of trinitrobenzenesulphonate before the addition of 10 mM-pyruvate for the exchange measurement. The rate for 14 mM-trinitrobenzenesulphonate was found to be identical if the preincubation time was extended to 60 min. The vertical bars represent the standard deviation as described for Fig. 3.

The reagent trinitrobenzenesulphonate, known to react with amino and thiol groups (Freedman & Radda, 1968) was the most powerful reagent tested for inhibiting the exchange in erythrocyte lysates, and the concentration-dependence of the inhibition (Fig. 4) indicates that there are approx. 20 μmol of exchange catalyst per ml of erythrocyte lysate. This strongly implies that residues of the haemoglobin molecule are responsible for the exchange, since the concentration of haemoglobin subunits in erythrocytes is approx. 20 mM. The failure of the thiol reagent *N*-ethylmaleimide to have any effect on the exchange (Table 1), in spite of its reactivity towards the free thiol groups in haemoglobin (Benesch & Benesch, 1961), supports the idea that amino groups are primarily responsible for catalysing the exchange.

Preparations of haemoglobin in ^2H_2O were found to exhibit exchange rates comparable with those of the erythrocyte, and it was found that the specific exchange-rate constant, expressed in terms of haemoglobin concentration, increases during the stages of haemoglobin purification. Values of $1.8\ (\pm0.2)\times10^{-4}$; $2.0\ (\pm0.2)\times10^{-4}$ and $2.8\ (\pm0.2)\times10^{-4}$/min per mg of haemoglobin per ml were found for erythrocyte lysates, $(NH_4)_2SO_4$-precipitated and DEAE–Sephadex purified haemoglobin respectively (see the Experimental section).

Several other proteins were examined to test for the exchange; from Table 2 it can be seen that some do, in fact, catalyse pyruvate proton exchange when present at concentrations comparable with that of haemoglobin in the erythrocyte. A notable exception is carbonic anhydrase, which differs from the others in not having a free α-amino group (Dayhoff, 1972).

Table 1. *Effect of various treatments on the exchange rate of pyruvate protons catalysed by (a) whole or (b) lysed erythrocytes in Krebs–Ringer buffer made with ^2H_2O and (c) haemoglobin preparations in ^2H_2O*

In all cases, 10 mM-pyruvate was added to the sample at time zero (see the Experimental section) and the rates were determined by logarithmic linear regression of the pyruvate peak height in $\tau = 60$ ms spin-echo spectra versus time. Results were corrected for packed cell volume or haemoglobin concentration.

Preparation	Treatment	Rate constant for proton exchange (% of untreated control = 100 ± 5%)
(*a*)	3 mM-Cycloserine present	100 ± 10
(*b*)	5 mM-Phosphoglycollate present	93 ± 5
	5 mM-Phosphoglycollate plus 2.5 mM-ADP present	105 ± 5
	Cells incubated for 1 h at 37°C with 30 mM-potassium cyanate, then lysed	62 ± 6
	Lysate preincubated for 0.5 h at 37°C with 12 mM-*N*-ethylmaleimide	100 ± 5
(*c*)	Deoxyhaemoglobin plus 10 mM-2,3-bisphosphoglycerate present	70 ± 6
	Oxyhaemoglobin preincubated for 1 h with 30 mM-potassium cyanate	35 ± 4

Table 2. *Rate constant for the exchange of pyruvate protons in ^2H_2O catalysed by some proteins*
The rate constant was determined by logarithmic linear regression of the pyruvate peak height in $\tau = 60$ ms spin-echo spectra versus time.

Protein	Concentration (mg/ml)	pH*	Rate constant for pyruvate hydrogen exchange (min^{-1})
Bovine carbonic anhydrase	200	7.5	0.002 ± 0.001
Bovine serum albumin	200	7.5	0.0086 ± 0.0009
Equine skeletal muscle myoglobin	200	7.5	0.020 ± 0.001
Hen egg-white lysozyme	60	8.5	0.011 ± 0.001

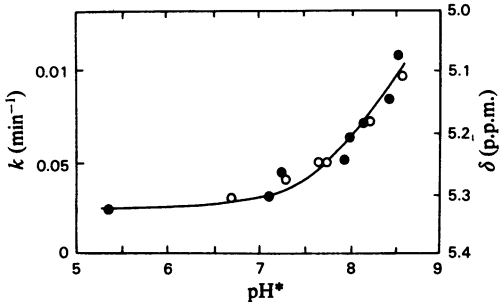

Fig. 5. *Pyruvate proton-exchange rate constant k* (●) *and chemical shift of the α-CH proton of lysine-1* (○) *of lysozyme (5 mM) in ^2H_2O versus pH**
For details see the Experimental section.

The pH*-dependence of the rate of the exchange in lysozyme solutions was investigated and the results are shown in Fig. 5. The titrating group has an apparent pK of about 8.5. Several reports of lysozyme pH titrations give only one group titrating in the pH range 7–10, namely the α-amino group (pK 7.8) (Imoto *et al.*, 1972). The pH* titration of the α-amino group was studied directly by observing the chemical shift of the α-CH resonance of lysine-1 (Campbell & Dobson, 1979). This yields the curve shown in Fig. 5, which corresponds well with the pyruvate-exchange-rate dependence on pH*.

The low levels of exchange observed with carbonic anhydrase (Table 2) and lysozyme at low pH* (Fig. 5) may correspond to the non-zero rate found with imidazole solutions [100 mM-imidazole in ^2H_2O, pH* 7.40, gives an exchange-rate constant of 0.0025 ($\pm$0.0007) min^{-1}]. Similarly, trinitrobenzenesulphonate-inhibited erythrocyte lysates yield a comparable baseline exchange rate (Fig. 4).

The evidence presented so far suggests that protein amino groups can catalyse pyruvate hydrogen exchange and that the predominant catalyst in erythrocytes is one or more amino group(s) of haemoglobin rather than an enzyme.

Studies by workers interested in inhibition of erythrocyte sickling (Acharya & Manning, 1980) and glycosylation of haemoglobin in diabetics (Bunn *et al.*, 1978) indicate that certain amino groups in human haemoglobin particiate in Schiff-base formation with sugar aldehydes. Such bond formation between these amino groups and the keto group of pyruvate may be expected to labilize the methyl hydrogens.

Some idea of the particular amino groups involved can be obtained from the results presented in Table 1. Carbamoylation of haemoglobin inhibits the exchange by 65% and the addition of 10 mM-2,3-bisphosphoglycerate to deoxyhaemoglobin produces a 30% inhibition. If erythrocytes are preincubated for 1 h with 30 mM-potassium cyanate before the pyruvate-exchange experiment, a procedure that leads to carbamoylation of roughly 1 α-amino group per haemoglobin tetramer (Cerami & Manning, 1971), a 38% inhibition of the exchange results (see Table 1).

The evidence presented suggests that the Val-1β amino group catalyses pyruvate isotope exchange, since it is in the 2,3-bisphosphoglycerate-binding site of deoxyhaemoglobin (Arnone, 1972). In addition, this group is reactive with cyanate (Garner *et al.*, 1975), trinitrobenzenesulphonate (Shinoda, 1965), glyceraldehyde (Acharya & Manning, 1980) and other sugar aldehydes (Bunn *et al.*, 1978). The involvement of Val-1α is also implied by its reactivity with trinitrobenzenesulphonate (Shinoda, 1965) and cyanate (Garner *et al.*, 1974) and the additional inhibitory effects of both these reagents on the exchange. The fact that extensive trinitrobenzenesulphonate treatment is required to give high inhibition implies that certain lysine ε-amino groups in haemoglobin (Shinoda, 1965) also catalyse the exchange.

In conclusion, therefore, the predominant catalysts of pyruvate–water isotope exchange in erythrocytes are the α-amino groups and one or more lysine ε-amino groups of haemoglobin. Other proteins in other cells are also expected to catalyse this exchange.

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