Transport of Phosphoenolpyruvate through the Erythrocyte Membrane

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Phosphoenolpyruvate was transported through the erythrocyte membrane at low pH (4.5–6.5). The influx was observed not only in an iso-osmotic sucrose medium, but also in 0.1 M-citrate solution, but it was negligible in an iso-osmotic NaCl solution. Efflux, however, was observed in both the sucrose and NaCl solutions. Compounds derived from phosphoenolpyruvate by replacing the methene group by similarly hydrophobic groups such as hydrogen or the methyl group were permeant but those with the hydrophilic hydroxymethyl group were impermeant. This transport was inhibited by the treatment with 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid or pyridoxal phosphate/ NaBH_4 , which are known to be specific for the transport of anions such as Cl^- , SO_4^{2-} and HPO_4^{2-} . It showed saturation kinetics with respect to phosphoenolpyruvate concentration in the medium. These results suggest that the transport of phosphoenolpyruvate is mediated by the anion-transport system. Although phosphoenolpyruvate was transported against the concentration gradient, the transport was characterized as a passive transport, and this apparent uphill transport was interpreted by the Donnan equilibrium.

Various kinetic studies on anion transport by human erythrocytes yielded two kinetic models of the transport, the fixed-charge model (Passow, 1969) and the mobile-carrier model (Gunn *et al.*, 1973). Successful application of specific inhibitors to anion transport in human erythrocyte (Cabantchik & Rothstein, 1972), together with theoretical and methodological advances in studies on the molecular architecture of the erythrocyte membrane (Bretscher, 1971; Fairbanks *et al.*, 1971; Singer & Nicolson, 1972), have greatly facilitated the progress of our knowledge on the mechanisms of anion transport. An involvement of a component in the cell membrane, the band-3 protein, in anion transport is now generally recognized (Cabantchik & Rothstein, 1974*a,b*; Rothstein *et al.*, 1975, 1976; Ross & McConnell, 1977).

Phosphorylated glycolytic intermediates have been usually considered not to penetrate the erythrocyte membrane. We reported, however, that phosphoenolpyruvate accumulated inside human erythrocytes in an acidified sucrose solution (pH 4.5) and suggested that the accumulation was due to its transport through the membrane (Tomoda *et al.*, 1975; Hamasaki *et al.*, 1977). The present report is concerned with the confirmation of its transport by the use of radioactive phosphoenolpyruvate. We used specific inhibitors of the anion-transport system and found that the phosphoenolpyruvate transport was closely related to the anion-transport system for Cl^- , SO_4^{2-} and HPO_4^{2-} . The transport was strongly dependent on the pH of the medium. The effect of pH on the anion-transport system was discussed.

Materials and Methods

Erythrocyte incubation

Human blood stored at 4°C in acid/citrate/dextrose solution was obtained from the Fukuoka Red Cross Blood Center. Erythrocytes stored for less than 2 weeks were usually used for experiments, but essentially the same results were obtained with cells stored for more than 2 weeks, although the rate of transport was diminished when cells stored for more than 1 month were used. The cells were washed thoroughly with 0.9% NaCl solution and washed with a sucrose medium (250 mM-sucrose, 10 mM-NaF, 10 mM-glucose). The washed cells were preincubated with a specified medium at 37°C for 10–30 min (haematocrit 5–15%). The incubation was started by the addition of 0.37 M-phosphoenolpyruvate and the pH of the incubation was adjusted by the addition of 0.1 M-HCl. Samples were taken at specified time intervals and the supernatant was separated by centrifugation for 0.5 min at room temperature and 7500g in an Eppendorf 3200 centrifuge. The erythrocyte fraction was resuspended in about 30 vol. of the cold sucrose medium and separated by the short centrifugation described above. Both the supernatant and the erythrocyte fractions were deproteinized with 0.6 M-HClO₄ or 10% (w/v) trichloroacetic acid. The extracts were kept in ice and used for enzymic assays within 2 h without neutralization. Intracellular concentrations of metabolites were calculated by assuming that free water occupies 70% of the cell volume (Beutler, 1971).

Analytical procedures

T.l.c. was done as described by Jeso (1968). Pyruvate and phosphoenolpyruvate were determined enzymically (Minakami *et al.*, 1965). Total phosphate and P_i was measured by the method of Fiske & SubbaRow (1925). Radioactivity was determined with a liquid-scintillation spectrometer (Horiba LS-501) with toluene/Triton X-100 (2:1, v/v) mixture containing 0.4% PPO (2,5-diphenyloxazole) and 0.01% dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene].

Reagents

All enzymes and glycolytic intermediates were from C. F. Boehringer und Soehne, Mannheim, Germany. Other reagents were of analytical reagent grade. Phospho[1- 14 C]enolpyruvate (14.4 mCi/mmol) and [1- 14 C]pyruvate (7.7 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and New England Nuclear, Boston, MA, U.S.A., respectively. 4-Acetamido-4'-isothiocyano-stilbene-2,2'-disulphonic acid was purchased from ICN Pharmaceuticals Inc., Cleveland, OH, U.S.A. Phenopyrazone was given by Dainippon Pharmacia Co., Osaka, Japan. 2-Phospholactate was kindly synthesized as the sesquibarium salt form by Daiichi Pure Chemicals Co., Tokyo, Japan.

Results

Transport of phosphoenolpyruvate into erythrocytes from a sucrose medium

Fig. 1 shows the changes in phosphoenolpyruvate concentrations in erythrocytes and in the suspending medium when the cells were incubated with 8.5 mM-phosphoenolpyruvate in a sucrose solution (250 mM-sucrose/10 mM-NaF/10 mM-glucose/10 mM-potassium phosphate, pH 6.0) at 37°C. The phosphoenolpyruvate concentration in the cells increased at the initial rate of 0.54 μ mol/min per ml of cells and reached 23 μ mol/ml of cells or 33 mM after 60 min incubation, whereas the phosphoenolpyruvate concentration in the medium decreased from 8.5 mM to 6.0 mM. The incubation was carried out in the presence of NaF, so that neither 2,3-bisphosphoglycerate nor monophosphoglycerates and only a little pyruvate were formed from phosphoenolpyruvate during the incubation, owing to the inhibitory effects of NaF on enolase (EC 4.2.1.11) and adenosine triphosphatase (EC 3.6.1.3). This is advantageous for quantitative measurement of the transport. An explanation for the apparent uphill accumulation of phosphoenolpyruvate against the concentration gradient is given below.

The changes of phosphoenolpyruvate concentration could also be calculated from the specific

Fig. 1. Transport of [1- 14 C]phosphoenolpyruvate through the human erythrocyte membrane at pH 6.1

Washed erythrocytes were preincubated in the sucrose medium (250 mM-sucrose, 10 mM-glucose, 10 mM-NaF) with 10 mM-potassium phosphate for 25 min at 37°C, pH 7 (haematocrit 6%) and the incubation was started by the addition of phospho[1- 14 C]enolpyruvate (2.3×10^4 c.p.m./ μ mol). The pH of the incubation was adjusted to 6.1 with 0.1 M-HCl. A sample of the erythrocyte suspension (0.5 ml) was taken out at the time specified, and the erythrocyte and supernatant fractions were prepared as described in the Materials and Methods section. Both fractions were treated with 0.6 M-HClO₄ and used within 2 h for enzymic and radioactive assays without neutralization. No significant haemolysis happened during incubation. The phosphoenolpyruvate concentration inside the cells was determined enzymically (○) and by radioactivity (●); △, extracellular concentration of phosphoenolpyruvate.

radioactivity of [1- 14 C]phosphoenolpyruvate and the radioactivities found in the cells and the medium. The results obtained by both enzymic assay of phosphoenolpyruvate and radioactivity were essentially in good agreement, as shown in Fig. 1, indicating that the accumulated phosphoenolpyruvate comes neither from glucose nor from any erythrocyte metabolite such as 2,3-bisphosphoglycerate, but from phosphoenolpyruvate of the medium.

Identification of radioactive compounds in erythrocytes

To show that phosphoenolpyruvate itself penetrates the erythrocyte membrane, the following radioisotope experiments were carried out (Table 1). When the deproteinized extract from washed erythro-

Table 1. *Distribution of ^{14}C radioactivity extracted from the erythrocyte fractions on t.l.c.*

Erythrocytes were incubated with 12mM-phospho-[1- ^{14}C]enolpyruvate (2.3×10^4 c.p.m./ μmol) (Expt. 1) or with 0.4mM-[1- ^{14}C]pyruvate (6.3×10^5 c.p.m./ μmol) and 8.5mM-non-radioactive phosphoenolpyruvate (Expt. 2) for 30min. Other experimental conditions are the same as in Fig. 1. The erythrocyte fractions were prepared as described in the Materials and Methods section. The HClO_4 extracts were neutralized by 5M-KOH to minimize the salt content of these extracts. Samples (5 μl) of the neutralized specimens were applied on a cellulose thin layer (300 μm thick) with non-radioactive pyruvate and phosphoenolpyruvate as a carrier, and developed with aq. NH_3 (28%)/methanol (3:7, v/v). Authentic phospho[1- ^{14}C]enolpyruvate was also treated with HClO_4 and KOH and applied on the same plate. Pyruvate and phosphoenolpyruvate were detected by the semicarbazide reagent (Umbarger & Magasanik, 1952) and by the molybdate reagent (Hanes & Isherwood, 1949) respectively. Chromatograms were almost equally divided into six fractions, in which fractions 2 and 5 corresponded to phosphoenolpyruvate and pyruvate respectively. The strip of each fraction was scraped off the plate and used for assay of radioactivity. General procedures for t.l.c. were described by Jeso (1968).

Fraction	Distribution of ^{14}C (%)		
	Authentic phospho-[^{14}C]enolpyruvate	Expt. 1 with phospho-[^{14}C]enolpyruvate	Expt. 2 with [^{14}C]pyruvate
1 (origin)	3.5	4.5	0.5
2 (phosphoenolpyruvate)	86.5	77.7	0
3	9.2	4.6	2.5
4	0	1.4	3.1
5	0.8	11.8	92.7
6	0	0	1.2

cytes incubated in the sucrose medium with phospho-[1- ^{14}C]enolpyruvate was subjected to t.l.c., most of the radioactivity was found in the phosphoenolpyruvate fraction. About 10% of the radioactivity was found in the pyruvate fraction, but this may either be due to the decomposition of phosphoenolpyruvate in the incubation medium or to conversion by the pyruvate kinase (EC 2.7.1.40) in the cells. A slight but significant increase in the intracellular pyruvate concentration was also observed by the enzymic assay. A thermodynamically less plausible possibility, that the radioactive phosphoenolpyruvate was formed from pyruvate in the cells, could be eliminated from the experiment in which erythrocytes were incubated with [1- ^{14}C]pyruvate and non-radioactive phosphoenolpyruvate, in which no radioactivity was found in the phosphoenolpyruvate fraction of the chromatogram.

Some properties of the phosphoenolpyruvate transport

The influx rate of phosphoenolpyruvate into erythrocytes incubated in the sucrose medium apparently followed saturation kinetics. A half-maximal velocity of phosphoenolpyruvate concentration was about 8mM (Fig. 2a). The rate was greatly influenced by the pH of the medium, steadily decreasing as the pH increased from 4.5 to 7.4. Essentially no transport was observed when the cells were incubated in the sucrose medium, pH 7.4, for 90min at 37°C (Fig. 2b). An Arrhenius plot showed the activation energy (ΔH) of the phenomenon to be 138 kJ/mol (33 kcal/mol) (Fig. 2c). The phosphoenolpyruvate influx was also observed when the sucrose medium was replaced by a citrate solution (100mM-citrate, 10mM-NaF, 10mM-potassium phosphate, pH 5.8), but negligible phosphoenolpyruvate influx was observed in a NaCl medium (150mM-NaCl, 10mM-NaF, 10mM-potassium phosphate, pH 6.0), as shown in Fig. 3.

The efflux of phosphoenolpyruvate from the erythrocytes preloaded with a high concentration of phosphoenolpyruvate was observed when the cells were resuspended in various phosphoenolpyruvate-free media (Fig. 4). The appearance of phosphoenolpyruvate in the media and its concomitant disappearance from the cells were shown by both enzymic and radioactive methods. Phosphoenolpyruvate efflux from erythrocytes incubated in the NaCl medium was observed, though the influx was negligible in this medium. The efflux rate from erythrocytes suspended in the sucrose medium was of similar magnitude to the influx rate.

Inhibition by the inhibitors of anion transport

The inhibitor of anion transport, 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonic acid (SITS) (Cabantchik & Rothstein, 1972), completely inhibited phosphoenolpyruvate transport (Fig. 5). The inhibitor for the ionic form of organic acids, phenylpyrazone (Deuticke, 1973), was effective, whereas the inhibitor for the transport of the non-ionic form of the acids, acetazolamide (Deuticke, 1973), had no significant effect on the phosphoenolpyruvate transport. Further, the pretreatment of erythrocytes with pyridoxal phosphate and NaBH_4 , which is known to inhibit anion transport specifically (Cabantchik *et al.*, 1975), also inhibited the phosphoenolpyruvate transport (Table 2). These observations may strongly suggest that phosphoenolpyruvate transport is mediated by the same anion-transport protein as for Cl^- , SO_4^{2-} , HPO_4^{2-} etc. 4-Acetamido-4'-isothiocyano-stilbene-2,2'-disulphonic acid inhibited not only the influx of phosphoenolpyruvate but also the efflux.

Fig. 2. Properties of phosphoenolpyruvate transport

(a) Effect of extracellular phosphoenolpyruvate concentration on the rate of its influx: erythrocytes were incubated in the sucrose medium with various concentrations of non-radioactive phosphoenolpyruvate at pH 6.0, 37°C (haematocrit 11%). To keep the ionic strength constant, total phosphate (potassium phosphoenolpyruvate and potassium phosphate) concentration in the incubation medium was fixed at 24 mM by replacing the P_i . The influx rate was calculated by the disappearance of phosphoenolpyruvate from the supernatant fraction. Other experimental conditions are the same as in Fig. 1. A half-maximal velocity of phosphoenolpyruvate concentration was obtained at about 8 mM by replotting of s/v against s (inset). (b) Effect of extracellular pH on the phosphoenolpyruvate influx rate: erythrocytes were incubated in the sucrose medium with 6.9–7.9 mM-phosphoenolpyruvate at 37°C (haematocrit 11%). The pH of the erythrocyte suspension was adjusted by 0.1M-HCl. The relative rate was calculated by the disappearance of phosphoenolpyruvate from the supernatant fraction at pH 4.5. Other experimental conditions are the same as in Fig. 1. (c) Arrhenius plot for the phosphoenolpyruvate influx rate: erythrocytes were preincubated in the sucrose medium with 10 mM-potassium phosphate for 20 min at about pH 7, 37°C . Incubations were started by the addition of 7.5–7.9 mM-non-radioactive phosphoenolpyruvate at pH 6.0 (haematocrit 6%) after the temperature of the erythrocyte suspensions was equilibrated. Experimental details are as shown in Fig. 1. The rate ($\mu\text{mol/min per ml}$ of cells) was determined by the changes of phosphoenolpyruvate in the both fractions.

Transport of other organic phosphate compounds

As previously described (Tomoda *et al.*, 1975; Hamasaki *et al.*, 1977), no accumulation of other glycolytic intermediates and nucleotides was observed when the cells were incubated with them in the sucrose medium. The metabolites included 3-phosphoglycerate, the molecular weight and pK_a of which are similar to those of phosphoenolpyruvate. Thus a series of other organic phosphate compounds were examined for their transport rate. As shown in Table 3, phosphoglycollate and 2-phospholactate could be transported through the membrane, whereas 2-phosphoglycerate and glycerol 2-phosphate could not. This observation may indicate that the compounds derived from phosphoenolpyruvate by replacing the methylene group by a hydrogen atom or a methyl group are permeable, whereas those with a hydroxymethyl group are not.

Explanation for the apparent uphill transport

It is quite unlikely that any active transport process is involved in the phenomenon, because NaF or ouabain had no effect on the transport, but this apparent uphill transport was explained by the Donnan equilibrium. To test this possibility, erythrocytes were incubated in the sucrose medium with a low concentration (0.4 mM) of $[1-^{14}\text{C}]$ pyruvate and a high concentration (8.5 mM) of non-radioactive phosphoenolpyruvate at pH 6.2, 37°C . Table 4 shows the distribution of pyruvate and phosphoenolpyruvate inside and outside the cells during the incubation. The pyruvate transport was so fast that the equilibrium seemed to be reached in 1 min, whereas the phosphoenolpyruvate was not in equilibrium until 60–75 min. We may suppose that the distribution of the radioactivity of pyruvate and

Fig. 3. Effects of media on the phosphoenolpyruvate influx. Erythrocytes were incubated in specified media (○, 250 mM-sucrose/10 mM-NaF/10 mM-glucose; ●, 100 mM-citrate/10 mM-NaF/10 mM-potassium phosphate; △, 150 mM-NaCl/10 mM-NaF/10 mM-potassium phosphate) with 6.4–7.7 mM-phosphoenolpyruvate at pH 5.8–6.0, 37°C (haematocrit 5–10%). Other experimental details are as shown in Fig. 1.

Fig. 4. Effects of media on the phosphoenolpyruvate efflux. Erythrocytes with a high phosphoenolpyruvate concentration were prepared in the sucrose medium by incubation with 8 mM-phospho[1-¹⁴C]enolpyruvate (4.8×10^4 c.p.m./ μ mol) as described in Fig. 1 and resuspended in phosphoenolpyruvate-free media with or without 1.8 mM-4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonic acid at pH 6.0, 37°C (haematocrit 6%). Phosphoenolpyruvate inside the cells and in the media was determined enzymically and by radioactivity. Other experimental details are shown in Fig. 1. Erythrocytes were incubated in the sucrose medium with (●) or without (○) 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonic acid and in the NaCl medium with (▲) or without (△) 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonic acid.

phosphoenolpyruvate and ΔpH ($=\text{pH}_i - \text{pH}_e$) (pH_i is intracellular pH, pH_e is extracellular pH) follows the Donnan equation described below (i, inside; o, outside). Although a part of the radioactive pyruvate is metabolized to lactate by the lactate dehydrogenase (EC 1.1.1.27) reaction in erythrocytes, the specific radioactivity and the number of negative charges per mol do not change:

$$\begin{aligned}\Delta\text{pH} &= \log \frac{[\text{H}^+]_o}{[\text{H}^+]_i} = \log \frac{[\text{pyruvate}^-]_i}{[\text{pyruvate}^-]_o} \\ &= \log \frac{[\text{phosphoenolpyruvate}]_i^{1/n}}{[\text{phosphoenolpyruvate}]_o^{1/n}}\end{aligned}$$

(when n is the number of negative charges on phosphoenolpyruvate).

The distribution of pyruvate across the membrane can be used for the calculation of ΔpH across the membrane, which gives 0.80 pH unit after 1 min incubation, in fair agreement with a reported value of 0.9 unit obtained from distribution of 5,5-dimethyl-oxazolidine-2,3-dione (Tomoda *et al.*, 1977). The ratio $[\text{pyruvate}]_i/[\text{pyruvate}]_o$ decreased from 6.3 to 1.8 by further incubation, and this decrease may reflect the pH_i decrease due to the entrance of a large amount of phosphoenolpyruvate into the cells.

At the equilibrium (60–75 min), the ΔpH across the membrane could be calculated as 0.26 unit. A corresponding pH change was observed by the direct pH measurement of the lysate of the packed cells (results not shown). The ratio $[\text{phosphoenolpyruvate}]_i/[\text{phosphoenolpyruvate}]_o$ expected from the equation is 5.5 when n is assumed to be 3, and 3.6 if $n = 2$. Since the ratio observed in 60–75 min was 5.8, the phosphoenolpyruvate transport against the concentration gradient could reasonably be explained by the Donnan equilibrium.

Discussion

It has been generally accepted that phosphorylated glycolytic intermediates cannot penetrate biological membranes. Nevertheless, transport systems for phosphoenolpyruvate have been demonstrated in some kinds of cellular membranes (Robinson, 1971; McCoy & Doeg, 1975; Saier *et al.*, 1975). The

Fig. 5. Effects of inhibitors of anion transport on the phosphoenolpyruvate influx

Erythrocytes were preincubated in the sucrose medium with 1.6 mM-acetazolamide (●), 1.6 mM-phenopyrazone (△) or 1.3 mM-4-acetamido-4'-isothiocyano-2,2'-disulphonic acid (▲) for 10 min at pH 6, 37°C (haematocrit 10%) and the incubation was started by the addition of phosphoenolpyruvate at 5.3–6.4 mM as an initial concentration. Erythrocytes incubated with 0.16% (v/v) dimethyl sulfoxide, which was used as a solvent for phenopyrazone, were used for a control experiment (○). Other experimental details are shown in Fig. 1.

Table 2. Effect of the pretreatment of erythrocytes with pyridoxal phosphate/NaBH₄ on the phosphoenolpyruvate influx rate

The treatment with pyridoxal phosphate/NaBH₄ was done as described by Cabantchik *et al.* (1975). The treated cells were washed and incubated with 7.0 mM-phospho[1-¹⁴C]enolpyruvate (3.7×10^4 c.p.m./μmol). Experimental details are given in Fig. 1. The influx rate was calculated from the changes of radio-active phosphoenolpyruvate in the both fractions.

Treatment	Influx rate (μmol/min per ml of cells)	Relative rate (%)
None	0.77	100
20 mM-NaBH ₄	0.66	86
1 mM-Pyridoxal phosphate and 20 mM-NaBH ₄	0.39	51
2 mM-Pyridoxal phosphate and 20 mM-NaBH ₄	0.24	31

present report is the first demonstration that a phosphorylated glycolytic intermediate such as phosphoenolpyruvate can penetrate the erythrocyte membrane, although the conditions of the incubation are somewhat artificial. The transport could be ascribed to the direct penetration of phosphoenolpyruvate in which no metabolic process seems to be involved. Phosphoenolpyruvate transport in both directions, influx and efflux, was observed. The phenomenon was also observed in 0.1 M-citrate medium as well as in the sucrose medium.

It is natural to assume that a mediator, probably the anion-transport protein of the cell membrane, is involved in phosphoenolpyruvate transport, because the transport showed well-defined saturation kinetics. Since Cabantchik & Rothstein (1972) reported that 4,4'-di-isothiocyano-2,2'-disulphonic acid, a stilbene derivative, binds covalently to an erythrocyte membrane protein and inhibits the anion transport, considerable evidence has been accumulated that the band-3 protein of the membrane is involved in anion transport. Cousin & Motais (1976) suggested that Cl⁻ and organic anions shared the same transport system in ox erythrocytes. The present observation that the treatment with 4-acetamido-4'-isothiocyano-2,2'-disulphonic acid or pyridoxal phosphate inhibited the transport suggests that phosphoenolpyruvate is transported by the anion-transport proteins. It should be specifically mentioned that phosphorylated organic compounds which are derived from phosphoenolpyruvate by replacing the methene group by similarly hydrophobic group such as hydrogen or the methyl group were permeant, but those with the hydrophilic hydroxymethyl group were impermeant. This is in accord with the results obtained by Cabantchik & Rothstein (1972) on the inhibitory effects of the stilbene derivatives that the compounds with a hydroxyl group on one or both ends of them are less effective than those without the hydroxyl group, and a hydrophobic group on one end of the compound is necessary for their inhibitory effect. The band-3 protein is a transmembrane protein (Jenkins & Tanner, 1975), which is supposed to exist as a dimer or tetramer at neutral pH (Steck, 1972, 1974; Wang & Richards, 1974; Cabantchik & Rothstein, 1974a). Further, it has been reported that the band-3 protein aggregates and is clustered reversibly at low pH (Nicolson, 1973; Rothstein *et al.*, 1976). Accordingly we may assume that the phosphoenolpyruvate transport at low pH is due to structural changes in the anion-binding site by the pH-induced clustering, which may affect the pH profile observed for the phosphoenolpyruvate transport. Taking all these into consideration, the transport of phosphoenolpyruvate through the erythrocyte membrane can be characterized as a mediated transport by the anion-transport protein of erythrocytes.

Table 3. *Transport of other organic phosphate compounds*

Erythrocytes were preincubated in the sucrose medium for 30 min at pH 6, 37°C (haematocrit 10%) and the incubation was started by the addition of each organic phosphate at 8 mM. The incubation was adjusted to pH 6.0 with 0.1 M-HCl. A portion (1.0 ml) of the erythrocyte suspension was taken out at the time specified, and the erythrocyte and the supernatant fractions were prepared as described in Fig. 1. Both fractions were treated with 10% (w/v) trichloroacetic acid and assayed for P_i and organic phosphate by the method of Fiske & SubbaRow (1925). The rate was determined by the changes of organic phosphate in the both fractions. The rate is given in parentheses as μmol/min per ml of cells.

Table 4. *Distribution of pyruvate and phosphoenolpyruvate between inside and outside the cells*

Erythrocytes were incubated with 0.4 mM-[1-¹⁴C]pyruvate (6.3 × 10⁵ c.p.m./μmol) and 8.5 mM-non-radioactive phosphoenolpyruvate at 37°C (haematocrit 7%). Phosphoenolpyruvate concentration was determined enzymically and pyruvate was calculated by radioactivity. Other details are shown in Fig. 1.

Incubation time (min)	pH _e	[Pyruvate] _i (mM)	[Pyruvate] _o (mM)	[Pyruvate] _i /[pyruvate] _o
1	—	2.75	0.44	6.3
15	6.1	1.75	0.51	3.4
30	6.2	1.44	0.52	2.8
45	6.2	1.13	0.51	2.2
60	6.2	0.97	0.52	1.9
75	6.3	0.95	0.52	1.8

Incubation time (min)	[Phosphoenolpyruvate] _i (mM)	[Phosphoenolpyruvate] _o (mM)	[Phosphoenolpyruvate] _i /[phosphoenolpyruvate] _o
1	0.5	8.5	0.06
15	15.1	7.3	2.1
30	25.3	6.4	3.9
45	31.3	6.4	4.9
60	33.6	6.0	5.6
75	34.6	5.8	6.0

Although the presence of impermeant solutes such as sucrose or citrate is necessary for the uphill accumulation of phosphoenolpyruvate inside the cells and the accumulation can mostly be explained by the Donnan equilibrium, there remains another possibility that a part of the phosphoenolpyruvate in the cells is trapped by haemoglobin. There is no experimental observation on the binding affinity of

phosphoenolpyruvate to haemoglobin, but we may safely assume that the dissociation constant of the phosphoenolpyruvate-oxyhaemoglobin complex is not less than about 5 mM, which is based on the experimental data for 2,3-bisphosphoglycerate, 1,3-bisphosphoglycerate, ATP and ADP at pH 7.2, 37°C (Hamasaki & Rose, 1974). On this assumption, the free phosphoenolpyruvate concentration inside the

cells is calculated to be not less than 26 mM (from the value of 60 min incubation in Table 4), which is definitely higher than the phosphoenolpyruvate concentration in the medium (6.0 mM). Accordingly, we cannot explain this uphill transport only by the binding of phosphoenolpyruvate to haemoglobin, but we can explain most of this accumulation from the Donnan distribution of the anions.

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