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PYRUVATE FLUX INTO RESEALED GHOSTS FROM HUMAN ERYTHROCYTES

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SUMMARY

The kinetics of pyruvate transport across the isolated red blood cell membrane were studied by a simple and precise spectrophotometric method: following the oxidation of NADH via lactate dehydrogenase trapped within resealed ghosts. The initial rate of pyruvate entry was linear. Influx was limited by saturation at high pyruvate concentration. Pyruvate influx was greatly stimulated by increasing ionic strength in the outer but not the inner aqueous compartment. The K_m ranged from 15.0 mM at $\mu = 0.05$ to 3.7 mM at $\mu = 0.01$, while the V went from $0.611 \cdot 10^{-15}$ to $0.137 \cdot 10^{-15}$ mol · min^{-1} · ghost^{-1}. Ionic strength was shown to affect the translocation step and not pyruvate binding. The energy of activation of pyruvate flux into resealed ghosts was 25 kcal/mol, similar to that found in intact red blood cells. Inhibitors of pyruvate influx included such anions as thiocyanate, chloride, bicarbonate, α-cyanocinnamate, salicylate and ketomalonate (but not acetate); noncompetitive inhibitors were phloretin, 1-fluoro-2,4-dinitrobenzene, 4-acetamido-4′-isothiocyanate-stilbene-2,2′-disulfonic acid and o-phenanthroline/$CuSO_4$ mixtures. The last reagent, known to induce disulfide links in certain membrane proteins, blocked the ionic strength stimulation of pyruvate influx in this study.

INTRODUCTION

Despite recent important advances, the molecular mechanism of facilitated anion transport in the red cell is unknown. Models proposing pores lined with fixed charges [1–5] or mobile carriers [6–8] have been suggested, but the issue remains open. Recently, Gunn [6, 9] provided evidence that the flux of Cl^- and other anions from red cells was facilitated by certain titratable groups. Kaplan and Passow [10] and Schnell et al [11] showed an asymmetry (or membrane sidedness) in the inhibition of Cl^- flux by phlorizin. Cabantchik and Rothstein [12] and Ho and Guidotti [13] have shown a close correlation between the inhibition of sulfate and phosphate trans-

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Abbreviations: N_2ph-F, 1-fluoro-2,4-dinitrobenzene; SITS, 4-acetamido-4′-isothiocyanate-stilbene-2,2′-disulfonic acid.

port, respectively, and the labeling of band 3 (the predominant red cell membrane polypeptide [14]) by radioactive, covalent ligands.

Anion transport has generally been studied in intact erythrocytes using radioactive solutes. While useful in equilibrium studies, this approach appears unwieldy for kinetic analyses [15]. A simpler and more precise system was constructed using the anion pyruvate, which appears to enter erythrocytes at a moderate rate by facilitated diffusion [16]. Pyruvate flux during the first few seconds of transport could be continuously monitored spectrophotometrically [17, 18] by trapping lactate dehydrogenase and NADH within resealed ghosts. Each membrane surface could be addressed unilaterally and each aqueous compartment defined and controlled.

MATERIALS AND METHODS

Materials

Pyruvic, lactic, L-malic, D-malic, citric, L-tartaric, M-tartaric, D-tartaric, malonic, tartronic, ketomalonic and glyoxylic acids, N_2ph-F, NADH and NAD^+ were obtained from Sigma (St. Louis). Rabbit muscle lactate dehydrogenase (type XI and type II) and pig heart malate dehydrogenase were from Sigma. Aldrich (Milwaukee) supplied 3-hydroxypropionic, 2-chloropropionic, *p*-hydroxybenzoic, acrylic and glycolic acids. Sodium salicylate was from Merck (Rahway, N. J.) and α-cyanocinnamic acid was from Pfaltz and Bauer (Flushing, N. Y.). K and K Laboratories (Plainview, N.Y.) provided phloretin and phlorizin. 4-Acetamido-4′-isothiocyanate-stilbene-2, 2′-disulfonic acid (SITS) came from ICN (Cleveland, Ohio) and diazinedicarboxylic acid bis-dimethylamide (diamide) from Calbiochem (LaJolla, Calif.). All other reagents were of the best analytical grade available.

Red blood cells

Fresh and freshly outdated bank blood samples from hematologically normal adult donors were used interchangeably without apparent difference. Cells were prepared according to Fairbanks et al. [19].

Resealed ghosts

All procedures were performed at 0–4 °C and all centrifugations were for 10 min at 15 000 rev./min in a Sorvall SS-34 rotor, unless indicated otherwise. Each ml of packed, washed red cells was lysed in 40 ml of ice-cold 5 mM sodium phosphate (pH 8) and sedimented for 10 min. The ghosts were incubated on ice for 5 min in an equal volume of 600 ideal mosM electrolyte solution, then resealed at 37 °C for 40 min. The resealed ghosts were recovered by centrifugation and washed three times into the buffer of interest. These ghosts retained approximately 1 % of their original hemoglobin. We found Mg^{2+} and Ca^{2+} to have no effect on the resealing process, except under conditions where chelators were present in the resealing suspension (e.g. citrate) at which time we usually included 0.1 mM Mg^{2+}.

We usually resealed the ghosts in NH_4Cl, a highly permeant electrolyte which could later be removed by washing. Resealed ghosts were filled with nonpermeating anions (e.g. malate) as required by substituting them equi-osmotically for NH_4Cl. We used sucrose as a nonpermeant nonelectrolyte; we routinely sealed 20 mM sucrose into ghosts to prevent their collapse in osmotically active media. We also added sucrose or another osmotically active solute to the external medium to prevent osmotic lysis of the resealed ghosts by the trapped sucrose. In a typical experiment, ghosts were resealed in an equal volume of 280 mM NH_4Cl plus 40 mM sucrose, washed once in 40 vols. of 380 mM sucrose (which shrinks the ghosts and removes the NH_4Cl), and then twice more in the buffer of interest.

Cells and ghosts were diluted 1 : 50 000 with a standard buffered saline solution and their numbers were estimated with a Model Z_B Coulter Counter.

Assay of pyruvate transport activity

Lactate dehydrogenase and NADH were trapped within ghosts by their inclusion in resealing buffer. After washing, resealed ghosts were resuspended 1 : 1 (v/v) in the buffer of interest (to approx. $3.5 \cdot 10^9$ ghost/ml) and 0.1 ml was briskly mixed with 0.9 ml of ammonium pyruvate solution in a semimicrocuvette. Absorbance at 340 nm was recorded as a function of time, employing a Gilford Model 240 spectrophotometer and a Health Model EU-205B recorder. Cuvette temperature was controlled by a Lauda K/2RD circulating water bath and was monitored by a Yellow Springs Instruments Telethermometer 43TD and Thermistor Probe Model 427. The pH of all test solutions was determined before and after each assay with a Radiometer PHM 64 pH meter. To destroy the permeability barrier, resealed ghosts were incubated prior to assay with one-tenth volume of 10 % Triton X-100 (Rohm and Haas).

Analysis of kinetic data was performed on constant initial rates using the nonlinear regression technique of Wilkinson [20].

Ionic strength (μ) was calculated from molar concentrations, assuming ideality, as $\mu = \Sigma Z_i^2 c_i/2$, where Z_i is the valency and c_i the concentration of the *i*th species.

Resealed ghosts were prepared and used the same day due to the lability of the NADH.

Red cell hemolysis tests

Isotonic salts of the highly permeable ammonium cation lyse red blood cells as a function of the rate of penetration of the counter anion [7, 21, 22]. To measure this process, 10 μl of packed red blood cells were suspended in 1 ml of isotonic ammonium salt solution in a thermostatted cuvette and absorbance at 610 nm was recorded. Half-times for lysis were calculated from the abrupt decrease in absorbance, according to Aubert and Motais [21]. Another method was to mix 1 ml of packed red cells thoroughly with 40 ml of the isotonic ammonium salt solution. Aliquots were taken at timed intervals, centrifuged and the release of hemoglobin, measured by its absorbance at 412 nm, taken as a measure of lysis. There was no discrepancy between the methods.

RESULTS

Characteristics of the pyruvate transport system

The two major rate-limiting steps in this system are the permeation of pyruvate through the membrane and the rate of NADH oxidation via lactate dehydrogenase. Fig. 1 demonstrates that a complete system (ghosts containing NADH plus lactate

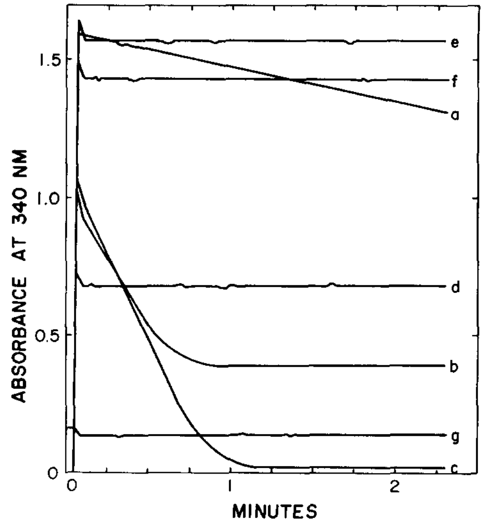

Fig. 1. Time course of pyruvate transport. The influence of several independent variables on the course of NADH oxidation was determined. (a) Complete system. Resealed ghosts containing lactate dehydrogenase and NADH were diluted to $2.2 \cdot 10^9$ ghosts/ml in 80 mM sucrose+10 mM ammonium acetate (pH 7.4). 0.10 ml was briskly mixed with 0.9 ml of 10 mM ammonium pyruvate +80 mM sucrose (pH 7.3) and absorbance followed at 340 nm, 17 °C. (b) Lysed ghosts. As in (a), except that the resealed ghost suspension was made 0.9 % in Triton X-100 before addition to the pyruvate solution. In (c), NADH and lactate dehydrogenase were added without ghosts. Ghosts added in (d) contained no NADH or lactate dehydrogenase and those added in (e) contained no lactate dehydrogenase. In (f), pyruvate was absent from the cuvette, while in (g) ghosts were omitted.

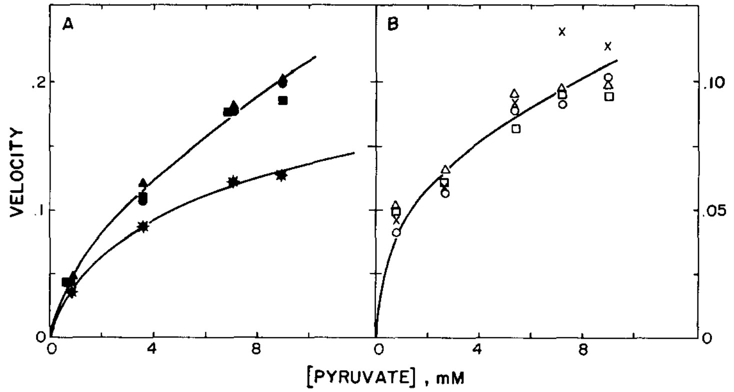

Fig. 2. Dependence of transport velocity upon lactate dehydrogenase and NADH concentration. (A) Ghosts were resealed in the presence of 10 mM NADH and lactate dehydrogenase at 40 (*), 80 (●), 120 (▲) and 160 (■) units/ml. Assays were performed as a function of pyruvate concentration at pH 7.8 and 16 °C. $\mu = 0.05$ was maintained with ammonium acetate. The external sucrose concentration was 80 mM. (B) as in (A), except that ghosts were resealed in the presence of 100 units lactate dehydrogenase/ml; NADH was 10.3 (×), 13.7 (○), 17.5 (△) and 22.6 (□) mM; assay was at 16 °C, pH 7.6, $\mu = 0.01$. Velocities for Figs. 2–9 are expressed as $\text{mol} \cdot \text{min}^{-1} \cdot \text{ghost}^{-1} \times 10^{15}$.

dehydrogenase; external medium containing pyruvate) was required for significant absorbance change. Variations in absorbance due to ghost settling and light scattering made a negligible contribution to rate measurements (Fig. 1, curves d and g). In principle, the time course would become nonlinear if (a) pyruvate were depleted, (b) NADH were depleted and/or (c) the lactate produced interfered with either NADH oxidation or the transport of pyruvate. The time course was linear from the outset (i.e. after the 5 s required to initiate the recording) until $\geqslant$ 40 % of the NADH had been utilized, indicating that these factors did not affect the initial rate measurement.

The rate of NADH oxidation increased hyperbolically with increasing pyruvate levels at constant ionic strength (Fig. 2A; velocities shown in this and all subsequent figures are in mol · min^{-1} · ghost^{-1} · 10^{15}). The level of lactate dehydrogenase was rate limiting at high pyruvate levels unless an input of 80–120 units lactate dehydrogenase/ml was utilized; then membrane permeability appeared to be rate determining at all pyruvate levels. NADH was not rate limiting if at least 10 mM was included in the resealing mixture (Fig. 2B), even at 50 mM pyruvate (not shown). By breaking the membrane barrier with 1 % Triton X-100, we demonstrated that lactate dehydrogenase and NADH levels in the ghosts were proportional to their concentration in the resealing medium and, furthermore, that the membrane was the principal rate-limiting factor in pryuvate reduction (Fig. 1). These data indicate that the membrane barrier can be made rate determining, so that NADH oxidation can be taken as a measure of pyruvate flux across the membrane. In subsequent experiments, the NADH and lactate dehydrogenase concentrations in the resealing suspension were 10 mM and 100 units/ml, respectively.

While we express velocities as mol · min^{-1} · ghost^{-1}, not all ghosts are resealed. However, previous experiments have shown that cells resealed in this manner are 90–100 % impermeable to small solutes such as NADH [23]. Some of the ghosts which remain leaky will not retain NADH and will not reduce pyruvate. Therefore, they will not appear as leaks in the present system, but will cause an underestimation of V. The fraction of the ghosts leaky to pyruvate is estimated below to be less than 2.5 % at low ionic strength.

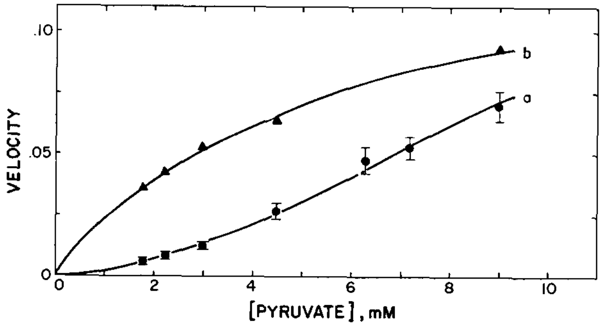

Fig. 3. Concentration dependence of pyruvate flux in the presence and absence of NH_4Cl. (a) Pyruvate influx was measured at 14.0 °C and pH 7.1 in the presence of 80 mM sucrose but no added electrolytes. (b) As in (a), except for the presence of 60 mM sucrose and 10 mM NH_4Cl, pH 7.4, throughout. Error bars indicate ±1 standard deviation for three independent experiments.

Stimulation of pyruvate influx by electrolytes

In the absence of other electrolytes, the dependence of transport rate on ammonium pyruvate concentration showed an unexpected sigmoid profile (Fig. 3, curve a). This pattern is not caused by stimulation from increasing levels of NH_4^+, since we observed similar sigmoid behavior employing sodium rather than ammonium pyruvate (not shown). The presence of other salts (e.g. NH_4Cl in Fig. 3, curve b) stimulated pyruvate influx by converting its concentration dependence to a hyperbolic form (see also Fig. 2A). At a constant pyruvate level, a variety of electrolytes stimulated substrate influx (Fig. 4), although some also produced inhibition at high concentrations. These electrolytes had little or no effect on the activity of free lactate dehydrogenase, nor was the stimulation attributable to the Na^+, since ammonium salts produced the same effect. Stimulation did not require a permeable anion, since sodium citrate and tartrate were more potent stimulators of pyruvate transport than NaCl or $NaHCO_3$, when present at the same concentrations. Stimulation lacked stereospecificity, as the sodium salts of M-, D- and L-tartaric acid were all equally active.

Fig. 5 indicates that stimulation of pyruvate by electrolytes is apparently a simple, saturable function of the ionic strength, which does not depend appreciably

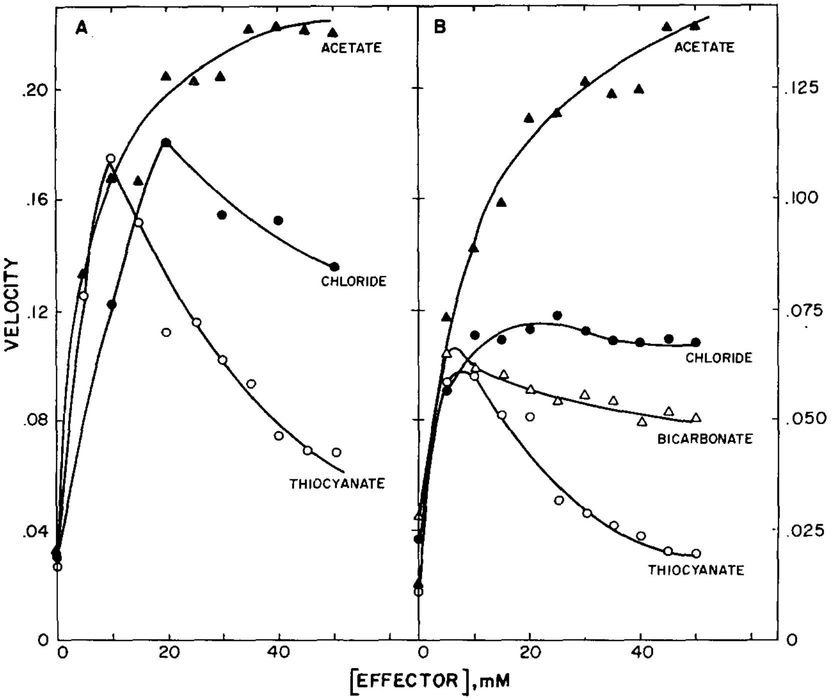

Fig. 4. Effect of electrolytes on pyruvate transport. Pyruvate influx was assayed at pH 7.6 in solutions containing 0.9 mM ammonium pyruvate, 80 mM sucrose and the indicated levels of the designated sodium salts at 37 °C (A) and 27 °C (B). To prevent osmotic artifacts at high salt concentrations, it was necessary to pre-incubate the resealed ghosts in an equal volume of double-strength salt solution.

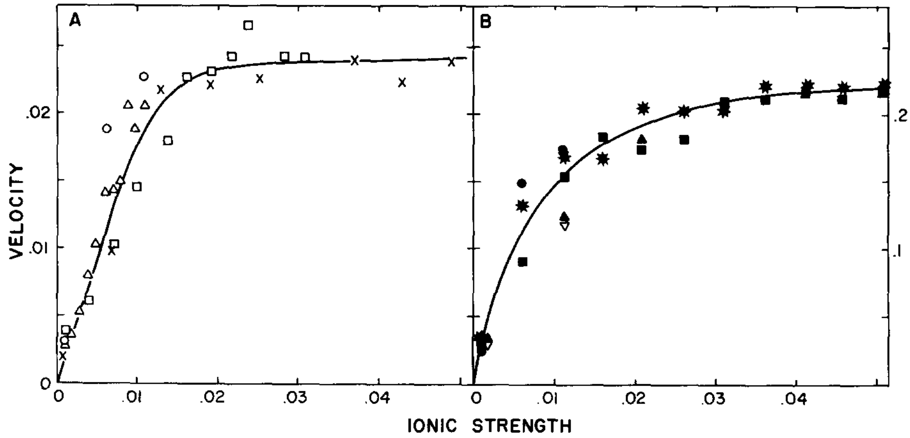

Fig. 5. The effect of ionic strength upon pyruvate flux. As in Fig. 4, except that the temperatures were 15 °C (A) and 37 °C (B). In (A), citrate (×), sulfate (□), bicarbonate (○) and chloride (△) In (B), acetate (★), formate (■), bicarbonate (▽), chloride (▲), and thiocyanate (●). To demonstrate more clearly the ionic activation effect of various electrolytes, data points in the inhibitory range of the last three salts (Fig. 4) were omitted from these plots.

upon the nature of the salt. This inference is supported by our observation that glycine produced little or no stimulation at high concentrations ($\geqslant$ 20 mM); this is attributed to the zero ionic strength of the zwitterion.

To avoid the complex dependence of rate on concentration, experiments were generally carried out at constant ionic strength, balancing ammonium pyruvate with acetate (as in Fig. 2), The latter anion strongly stimulates influx but causes little or no inhibition (Fig. 4), presumably because it does not utilize the anion carrier [16].

Table I presents kinetic values for pyruvate influx as a function of ionic strenght. Both K_m and V dropped with decreasing μ, while K_m/V remained nearly constant. Although the data shown are from a single experiment, similar values were obtained with at least three other preparations and fell within 1 standard deviation of those cited. The data did not vary with the serotype, source or age of the blood.

To assess possible sidedness (asymmetry) of the ionic stimulation of pyruvate

TABLE I

EFFECT OF IONIC STRENGTH UPON KINETIC PARAMETERS

Kinetic analysis of pyruvate influx was performed at 17 °C and a pH of 7.6. Ionic strength levels were maintained by mixtures of ammonium pyruvate plus acetate. External sucrose was 80 mM.

μ	K_m (mM)	V (mol · min^{-1} · ghost^{-1}) × 10^{15}	K_m/V (min · ghost · L^{-1}) × 10^{-12}	k_2* (min^{-1})
0.050	15.0±1.2	0.611±0.021	24.5±2.8	1230
0.025	4.8±0.9	0.197±0.011	24.4±5.9	396
0.010	3.7±0.4	0.137±0.006	27.0±3.5	275

* k_2 was calculated as V/E_0, assuming 300 000 anion transport sites per ghost [12, 13].

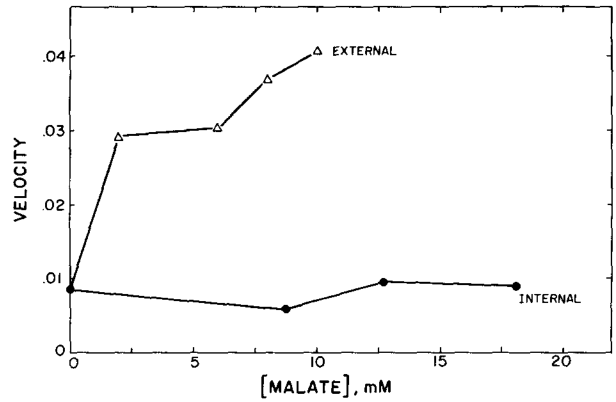

Fig. 6. Asymmetry of malate activation. Pyruvate influx was assayed at 18.0° and pH 7.4 in the presence of 0.9 mM ammonium pyruvate and 80 mM sucrose. The internal malate concentration was varied with no malate present externally and the external malate concentration was varied with no malate present internally. The concentration of malate trapped inside resealed ghosts was determined as follows. A calibration curve was constructed by adding 0.020 ml aliquots of 0–20 mM malate to cuvettes containing 1 ml of 2.5 mM NAD$^+$, 10 units malate dehydrogenase and 100 mM glycine (adjusted to pH 10.2 with NaOH). The total absorbance change was measured and found to be proportional to initial malate concentration. Ghosts containing malate were lysed with Triton X-100 and 20 μl assayed as above and quantitated using the standard curve determined simultaneously.

influx, resealed ghosts were prepared in varying quantities of the impermeant anion, malate (Fig. 6). While 10 mM malate, present externally, markedly stimulated uptake rate, up to 20 mM malate on the inside of the ghosts had no significant effect on pyruvate influx. Up to 40 mM malate did not affect the K_m of free lactate dehydrogenase; it reduced V by 15 %, but this effect cannot explain the apparent asymmetrical stimulation of the pyruvate influx observed.

Citrate, another impermeable anion, showed the same asymmetric stimulation as malate; however, its concentration within resealed ghosts was not directly measured, as was malate.

Inhibition of pyruvate influx by other anions

Fig. 4 revealed that high concentrations of certain salts inhibited pyruvate influx. We therefore examined the inhibitory effects of a variety of anions, including several pyruvate analogues, and compared these results with their ability to cross the cell membrane (as measured by red cell lysis time). These findings are summarized in Table II.

With respect to lysis times, the anions fall into two main groups: (a) those which lysed cells in less than 2 h; and (b) those which did not lyse cells during the 21 h experiment. Those in the latter category contained at least a four-carbon backbone and two carboxylic groups, consistent with previous findings [21]. Salts of dicarboxylic acids with a three-carbon backbone (for example, malonate) were readily permeable. While inexplicable, it is important to realize that pyruvate analogues were not distinctly better inhibitors of its uptake than seemingly unrelated anions. The best inhibitors were substituted aromatic anions (Table II), as was found earlier

TABLE II

A COMPARISON OF THE INHIBITORY EFFECT OF VARIOUS ANIONS ON PYRUVATE INFLUX WITH THEIR HEMOLYTIC EFFICIENCY

The inhibition of pyruvate influx by the sodium salts of the indicated anions was measured at 17.0 °C and pH 7.3. The pyruvate, ammonium acetate and external sucrose concentrations were 0.9 mM, 25 mM and 80 mM, respectively. Lysis experiments were carried out at the same temperature and pH, using isosmotic ammonium salts of the indicated anions. The $t_{\frac{1}{2}}$ was determined as indicated in Methods.

Concentration producing 50 % inhibition (mM)	Anion	$t_{\frac{1}{2}}$ (min)
< 10	Salicylate	< 40
	p-hydroxybenzoate	< 40
25–50	Thiocyanate	< 40
	Ketomalonate*	< 100
70–110	Iodide	< 5
	Bicarbonate	< 5
> 110	Chloride	< 5
	Formate	< 5
	Tartronate*	< 100
No effect	Malonate	< 70
	2-chloropropionate*	< 100
	3-hydroxypropionate*	< 100
Slight stimulation (< 10 %)	L-Tartrate	> 1260
	D-Tartrate	> 1260
	Citrate	> 1260
	Malate	> 1260
	Acetate	< 5

* Pyruvate analog.

for Cl^- and SO_4^{2-} flux in intact red cells [24, 25]. We have found that the anions inhibitory of pyruvate flux were those able to cross the membrane. That is, those anions whose ammonium salt did not lyse the cells did not inhibit pyruvate flux; however, not all lytic anions were good inhibitors (e.g. acetate, which is able to cross the membrane as the undissociated acid [16]).

α-Cyanocinnamate and phloretin were found to be noncompetitive inhibitors of pyruvate influx. The former compound, known to inhibit pyruvate flux in intact cells [26, 27], produced 70 % inhibition of V when present at 2 mM in the external aqueous compartment. Phloretin, which inhibits Cl^- flux in the red cell [24], produced 70 %, 57 % and 36 % inhibition of V at $2.5 \cdot 10^{-4}$, $1.25 \cdot 10^{-4}$ and $2.5 \cdot 10^{-5}$ M, respectively.

Use of chloride to vary the ionic strength

When Cl^- was used to maintain constant ionic strength, a series of convex velocity vs. substrate curves was obtained at three ionic strengths (Fig. 7). Identical results were obtained with formate. We suspect that the convexity was the result of

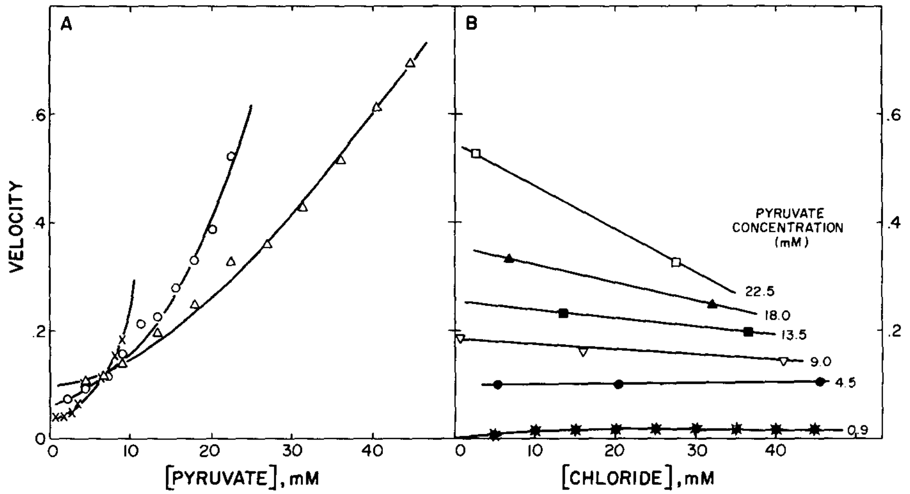

Fig. 7. Stimulation and inhibition of pyruvate influx by ammonium chloride. (A) Pyruvate influx was assayed at pH 7.7 and 17 °C at μ = 0.010 (×), 0.025 (○) and 0.050 (△), by varying the ammonium pyruvate and Cl^- concentrations. (B) Data from (A) were replotted. The 0.9 mM pyruvate data were obtained in a separate experiment, as in Fig. 4.

removing a competitive anion (Cl^-) at the same time substrate concentration was being increased. Qualitatively similar results were obtained by Deuticke [28], who varied Cl^- and phosphate concentrations reciprocally and by Gunn et al. [29], who varied Cl^- and HCO_3^- concentrations simultaneously.

Replotting velocities from Fig. 7A as a function of Cl^- concentration at constant pyruvate levels (Fig. 7B) demonstrated Cl^- inhibition of pyruvate influx at elevated levels of the substrate; however, stimulation occurred at low pyruvate levels. Inhibition by Cl^- was not obvious in earlier experiments at 17 °C (not shown) or 27 °C (Fig. 4). This behavior is predicted from kinetic theory, assuming that the effector (Cl^-) acted as both an activator and a competitor. We conclude that Cl^- is an inhibitor of pyruvate flux, even though inhibition is not apparent in certain types of experiments.

Mediated vs. nonmediated transport

We postulated that nonmediated entry of pyruvate into ghosts (for example, direct diffusion through holes) would not be stimulated by electrolytes, as would facilitated transport. Therefore, the stimulation of pyruvate influx by sodium acetate was studied. Relative stimulation by 50 mM acetate increased as the temperature decreased, from 5-fold at 37 °C to 13-fold at 17 °C. If nonmediated pyruvate flux is not affected by other ions, then at most 8 % of the pyruvate flux was not mediated under these conditions. Since pyruvate influx at low ionic strength was inhibited by 70 % with phloretin at $2.5 \cdot 10^{-4}$ M, the mediated pathway must still be dominant at this low ionic strength. Thus, the fraction of pyruvate entering the cells by a nonmediated route may be 2.5 % or less (i.e. 30 % of 8 %) of total influx under these conditions.

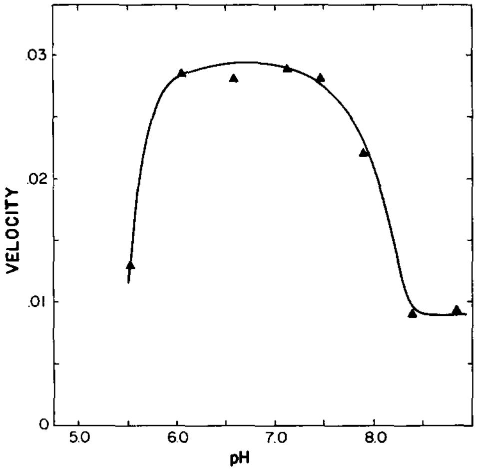

Fig. 8. pH dependence of pyruvate transport. Pyruvate influx was measured at 17 °C in 50 mM ammonium acetate, 80 mM sucrose and 0.9 mM ammonium pyruvate. The pH was varied by the addition of dilute NaOH and acetic acid. Since pyruvate levels were not saturating, this curve is representative but may not be a sole function of V.

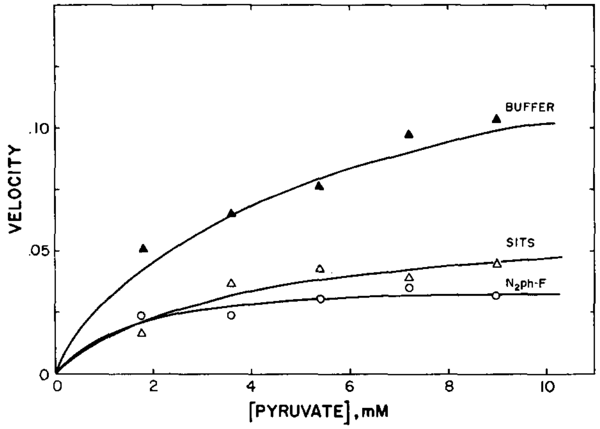

Fig. 9. Effect of SITS and N_2ph-F on pyruvate transport. Resealed ghosts were treated with buffer (80 mM sucrose+10 mM NH_4 acetate, pH 7.4) (▲), 5 mM N_2ph-F for 5 min at 37 °C (○), or 0.1 mM SITS for 30 min at 37 °C (△). The ghosts were then washed three times with buffer. Pyruvate influx was assayed at 17 °C (pH 7.5) in a medium containing 80 mM sucrose and varying amounts of ammonium pyruvate; ionic strength was maintained with ammonium acetate at 0.01. The kinetic parameters for these curves were: Buffer, $K_m = 4.8 \pm 1.7$ mM and $V = 0.1495 \pm 0.0241$; SITS, $K_m = 3.6 \pm 1.3$ mM and $V = 0.0627 \pm 0.0097$; N_2ph-F, $K_m = 1.36 \pm 0.75$ mM and $V = 0.0370 \pm 0.0048$.

Although the ammonium salt may react with these reagents, for technical reasons this protocol seemed most satisfactory and gave significant results.

pH dependence

Fig. 8 shows a broad optimum for pyruvate influx between pH 6.0 and 7.7. Control experiments demonstrated that the pH dependence of lactate dehydrogenase did not contribute to this pH profile.

Activation energy

The temperature dependence of the pyruvate influx between 7 and 37 °C at $\mu = 0.01$ was marked. Arrhenius analysis led to values of 25 kcal/mol for the activation energy, equivalent to a Q_{10} of 4.2. A similar study of free lactate dehydrogenase yielded a value of 3.3 kcal/mol ($Q_{10} = 1.2$) at a pyruvate concentration of 1 mM, further indication that transport across the membrane was rate limiting in the reaction of external pyruvate with internal lactate dehydrogenase.

Covalent modifiers

SITS, which binds both covalently and noncovalently to the red blood cell membrane [12, 30], and N_2ph-F, which acts via covalent modification [31–33], both inhibit pyruvate influx (Fig. 9). Although SITS produced a 30 % inhibition of V for free lactate dehydrogenase, this compound is not able to cross the membrane [12], so that the inhibition observed in Fig. 9 is most probably caused by its effect upon the transport system.

o-Phenanthroline/$CuSO_4$

o-Phenanthroline (100 μM) plus $CuSO_4$ (20 μM) were added to the resealing solution of freshly lysed ghosts. This chelate complex, which catalyzed the oxidation of membrane sulfhydryl groups to disulfide linkages [34, 35] did not perturb free lactate dehydrogenase significantly. However, this treatment reduced the K_m of pyruvate influx from 15.0 ± 1.2 mM to 1.7 ± 0.3 mM and reduced V from $0.611 \cdot 10^{-15} \pm 0.21 \cdot 10^{-15}$ to $0.200 \cdot 10^{-15} \pm 0.005 \cdot 10^{-15}$ mol · min^{-1} · ghost^{-1} at a constant ionic strength of 0.05. Similar kinetic values were obtained for *o*-phenanthroline/$CuSO_4$-treated resealed ghosts at $\mu = 0.01$ and $\mu = 0.025$. Since these values are similar to those obtained in untreated ghosts at low ionic strength, it appears that *o*-phenanthroline/$CuSO_4$ treatment blocks the ionic activation effect.

External *o*-phenanthroline/$CuSO_4$ had no effect on pyruvate flux, nor did *o*-phenanthroline or $CuSO_4$ alone. Another disulfide cross-linking agent, diazinedicarboxylic acid bis-dimethylamide (diamide), also had no effect on either K_m or V.

DISCUSSION

As previously shown for glucose [36], pyruvate flux into resealed red cell ghosts can be studied by means of a coupled enzyme system. At least 97 % of the influx observed under specified conditions appeared to be mediated and not free diffusion. That transport and not the enzyme reaction was rate limiting was demonstrated in Figs 1 and 2. When cells were lysed using Triton X-100, the initial rate increased 10-fold. Furthermore, the activation energy for the free enzyme reaction was 3 kcal/mol, compared to the value of 25 kcal/mol for intact ghost preparations.

This system shows close similarities to other anion transport systems of the intact red blood cell. (a) All systems show saturation kinetics and mutual competition among a variety of anions [25, 28, 29, 37]. (b) Deuticke [16] found values of 24–30 kcal/mol for the activation energy of the exchange flux of some organic anions with Cl^- in intact red blood cells, similar to our value of 25 kcal/mol. (c) A broad plateau of peak activity in the pH range 6.0–7.7 was observed here for pyruvate and by Gunn [6] for Cl^- and I^- self-exchange flux in red cells. (d) The present system was inhibited by SITS and N_2ph-F, inhibitors of $SO_4{}^{2-}$ and Cl^- flux in the intact red blood cell [12, 31, 33]. (e) α-Cyanocinnamate, a potent inhibitor of pyruvate flux in intact red cells [26, 27], was also very effective in the present system, as was phloretin, an inhibitor of Cl^- flux [24]. Thus, the pyruvate transport system studied in resealed ghosts is similar in all respects studied so far to that of intact red blood cells. The present experiments further support the premise that pyruvate shares a common transport system with Cl^- and other anions [16, 24, 28, 29, 33, 37].

Let us assume that the initial influx of pyruvate can be considered simplistically as consisting of a binding and a translocation step, analogous to an enzyme reaction. Then

$$\text{Pyruvate}+\text{T} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Pyruvate—T} \xrightarrow{k_2} \text{Pyruvate*} \tag{1}$$

where T is the translocator and * indicates the inner surface. Then,

$$K_m = \frac{k_{-1}+k_2}{k_1}$$

$$V = k_2E_0$$

$$K_m/V = \left(\frac{k_{-1}}{k_2}+1\right)\Big/E_0k_1.$$

Since K_m/V varies little with μ (Table I), both K_m and V must change simultaneously. k_2 is the only kinetic term which is a factor in both K_m and V; thus, ionic strength must largely affect k_2 and $k_2 \gg k_{-1}$. By plotting K_m versus k_2 for the data in Table I, we obtained values of $k_1 = 83.2\ \text{mM}^{-1}\cdot\text{min}^{-1}$, $k_{-1}/k_1 = 0.23$ mM and $k_{-1} = 19.1\ \text{min}^{-1}$. This analysis confirms the premise that k_2 is much larger than k_{-1} at $\mu > 0.01$ (see Table I). We have not ascertained whether k_2 falls to 0 at $\mu = 0$ (that is, if there is an absolute ionic strength requirement for pyruvate transport). Fig. 3 suggests that this may be true since, at 1 mM ammonium pyruvate, flux is 10-fold less at $\mu = 0.001$ than at $\mu = 0.011$.

The sigmoid dependence of pyruvate flux (Fig. 3, curve a) could reflect a simple higher order dependence on pyruvate concentration, homotropic allosteric cooperativity [38] or pyruvate binding to separate activator and substrate sites. Similarly, we cannot distinguish whether the ionic activation reflects binding to one of the substrate sites of a cooperative oligomer or whether there exist separate substrate and activator sites. Equivalent stimulation by all ions as a function of ionic strength (Fig. 5) despite their unequal inhibition (Table II) seems to support the latter choice. The inhibition produced by certain ions could be due to competition at the substrate site.

The ionic effect was not due to a change in membrane potential, since both permeable and non-permeable ions produced stimulation. The same ion (malate) produced different effects in the outer and inner aqueous compartments. Treatment of the inner compartment of resealed ghosts with *o*-phenanthroline/$CuSO_4$ inhibited ionic stimulation but not the basal rate. It may be significant that this reagent is known to crosslink band 3 selectively at the cytoplasmic surface [34, 35]. Band 3 is the predominant membrane spanning polypeptide [14, 39–42] which has been implicated in anion transport [12, 13].

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We have recently prepared hemoglobin-free, resealed inside-out vesicles with lactate dehydrogenase and NADH inside and have characterized pyruvate flux into these vesicles. It is likely that this assay method will be useful in the study of transport into liposomes reconstituted from purified components.

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