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PYRUVATE TRANSPORT INTO INSIDE-OUT VESICLES ISOLATED FROM HUMAN ERYTHROCYTE MEMBRANES

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Summary

1. A method has been developed for measuring the facilitated diffusion of pyruvate anions across the isolated human erythrocyte membrane. Ghosts were resealed in the presence of an excess of lactate dehydrogenase and NADH so that the influx of pyruvate could be followed by a continuous spectrophotometric recording of the oxidation of NADH (Rice, W.R. and Steck, T.L. (1976) Biochim. Biophys. Acta 433, 39—53). This approach has now been extended to sealed inside-out vesicles derived from the same membrane.

2. Transport across inside-out and right-side-out membranes was similar in certain respects. In both cases, the influx of pyruvate was greatly stimulated by increasing the ionic strength in the external medium. Salts of transported anions (such as chloride) but not non-transported anions (such as malate or acetate) were inhibitory at high concentrations, presumably a competitive effect. The non-competitive inhibitors of anion flux, salicylate, 4-acetamido-4′-isothiocyanato-2,2′-stilbene disulfonate (SITS) and 1-fluoro-2,4-dinitrobenzene, reduced pyruvate transport from both sides of the membrane.

3. Characteristic asymmetries in the transport of pyruvate were noted. Probenecid was found to act only at the cytoplasmic surface, although it inhibited flux in both directions. Mixtures of *o*-phenanthroline and $CuSO_4$ catalyzed the disulfide cross-linking of band 3, the purported anion transport protein, only at the membrane's cytoplasmic surface. This treatment stimulated pyruvate flux into resealed ghosts, but inhibited flux into inside-out vesicles.

4. The binding of glyceraldehyde-3-phosphate dehydrogenase and aldolase to the cytoplasmic surface of the membrane did not significantly alter pyruvate flux in either direction, even though these enzymes are known to associate specifically with band 3. Proteolytic excision of the cytoplasmic domain of band 3, amounting to approximately 40% of its mass, also had no effect on pyruvate flux.

Introduction

Structural studies on the human erythrocyte membrane have indicated a strong asymmetry in the distribution of proteins between the two membrane faces (cf. refs. 1 and 2). While the active transport of solutes is clearly anisotropic [3—8], the mechanism for the facilitated diffusion of solutes has in the past been viewed as involving a symmetrical carrier which diffuses through the membrane between the two surfaces (cf. ref. 9). However, there is growing evidence that the facilitated diffusion of anions [10,11] and glucose [12—15] in this membrane is also asymmetric. Furthermore, recent studies in several laboratories [16—18] have suggested that anion transport in the red cell is mediated by band 3, a membrane-spanning glycoprotein of molecular weight approx. 90 000 which is fixed asymmetrically in the membrane [1,19—22]. It is clear that the elucidation of the molecular basis of anion transport will entail structural and kinetic studies at both the outer and inner membrane surfaces.

We have developed an approach to the study of the transport of the organic anion, pyruvate, through the isolated erythrocyte membrane [23]. The assay couple, lactate dehydrogenase plus NADH, is trapped inside sealed ghosts, right-side-out vesicles or inside-out vesicles during their preparation. The flux of pyruvate into the sealed compartment can be made rate-limiting and can readily be followed spectrophotometrically as NADH is oxidized and pyruvate reduced to lactate. In this way, the initial flux (rather than the approach to exchange equilibrium) is measured directly, without separating the membranes from the medium. The use of isolated membranes, ghosts and vesicles, also allows better definition and control of the milieu in the internal compartment than can be achieved with intact erythrocytes.

Our initial studies of the transport of pyruvate into resealed ghosts were recently published [23]. We now present a complementary analysis of flux in the opposite direction, using inside-out vesicles. A preliminary account of this work has been reported [24].

Materials and Methods

Materials

Pyruvic, L-malic and D-malic acids, 1-fluoro-2,4-dinitrobenzene, and NADH were obtained from Sigma Chemical Co. (St. Louis, Mo.). We obtained sodium salicylate from Merck (Rahway, N.J.) and 4-acetamido-4′-isothiocyanato-2,2′-stilbene disulfonate (SITS) from ICN (Cleveland, Ohio). All other reagents were of the best analytical grade available. Rabbit muscle lactate dehydrogenase (type II or XI) was obtained from Sigma.

Membranes

Fresh and freshly outdated bank blood samples from hematologically normal adult donors were used interchangably with no systematic differences observed. Cells were washed and unsealed ghosts prepared according to published protocols [25—27]. Resealed ghosts were prepared for pyruvate transport studies as previously described [23]. Unless otherwise noted, membranes were prepared and maintained at 0—5° C and all centrifugations were for 10 min at 15 000 rev/min in a Sorvall SS-34 rotor. Cell and ghost numbers were estimated

with a Model Z_B Coulter Counter in a standard buffered saline solution at 1 : 50 000 dilution.

To prepare inside-out vesicles, 1-ml portions of packed unsealed ghosts were suspended in 40 ml of chilled, 0.5 mM sodium phosphate (pH 8) and incubated on ice for 30 min [26,27]. The membranes, primed for vesiculation, were collected by a 5 min centrifugation. At this point, the pellets typically contained 5% sealed right-side-out vesicles but no sealed inside-out vesicles. Sealed inside-out vesicles containing the assay couple were generated by resuspending pellets derived from 1 ml ghosts to a 1 ml volume containing (final concentrations): 0.5 mM sodium phosphate (pH 8), 40—200 U/ml lactate dehydrogenase, 0.5 mM NADH and 20 mM sucrose. The suspensions were stored overnight at 4° C. Each suspension was then agitated vigorously for 10 s on a vortex mixer and homogenized by expelling 3—5 times through a No. 27 gauge needle on a 1 ml syringe to generate sealed vesicles [26,27]. The vesicles were finally washed three times in the assay buffer.

The fraction of each preparation present as sealed vesicles of each orientation and as unsealed membranes was determined before and after the transport assays by the accessibility of acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase to their respective substrates [26,27]. The vesicles were found to be stable, with no evidence of breakdown or interconversion during storage or transport assay. A typical preparation contained approximately 50% sealed inside-out vesicles and 5% sealed right-side-out vesicles, the remainder being unsealed.

We typically determined the flux of pyruvate into inside-out vesicles in these unfractionated mixtures. We reasoned that unsealed vesicles should not contribute to the assay, since they do not retain NADH during the wash cycles. This premise was verified by purifying the sealed fraction from vesicle mixtures by dextran density gradient centrifugation [26,27]. The kinetics of transport did not change as the contaminating unsealed vesicles were reduced from 50 to 20% of the mixture. Similarly, we neglected the contamination by sealed right-side-out vesicles, since (a) this was not greater than 10% of the inside-out fraction (Table I), and (b) most of the right-side-out vesicles sealed prior to the addition of NADH and thus did not register in the assay.

Total membrane protein cannot be used to quantitatively relate transport data on vesicles to whole cells and resealed ghosts, since certain peripheral proteins are lost during the vesiculation process [26]. The intrinsic protein, acetylcholinesterase [2,28], however, can be used for such quantitation [26,27]. Data were therefore quantitated in ghost equivalents (i.e. that quantity of inside-out vesicles with the acetylcholinesterase activity of one ghost).

Sealed right-side-out vesicles containing the assay couple were prepared for pyruvate transport studies by following the procedure described above for inside-out vesicles, except that 0.1 mM $MgSO_4$ (final) was added to the ghost suspensions after the 30 min incubation in 0.5 mM sodium phosphate (just prior to the 5 min centrifugation) and to the resealing buffer as well (cf. ref. 26).

Assay of pyruvate transport

Sealed vesicles were generated in the presence of lactate dehydrogenase and

NADH, as described above. Because of the lability of NADH, vesicles were used only on the day they were prepared. The vesicles were washed and resuspended in an equal volume of the buffer of interest to approximately $7 \cdot 10^9$ ghost equivalents/ml (the concentration of packed ghosts). Aliquots of vesicle suspensions (0.1 ml) were mixed briskly with 0.9 ml of ammonium pyruvate solutions in a semi-microcuvette. The change in absorbance at 340 nm was recorded as a function of time during the linear interval, as described previously [23].

Lineweaver-Burk plots of constant (initial velocity)$^{-1}$ vs. (substrate concentration)$^{-1}$ were constructed. If linear, the data were further analyzed using the regression technique of Wilkinson [29] or the graphical method of Cornish-Bowden [30—32]. No significant differences between the parameters obtained by the two methods were noted. The abbreviations V_t and K_t designate the maximal velocity of transport under the conditions specified and the substrate concentration giving $V_t/2$, respectively. Superscripts E and C designate the extracellular and cytoplasmic surfaces of red blood cells, respectively. Thus, K_t^C and V_t^C refer to flux from the external to the internal compartment of inside-out vesicles. Velocities are expressed throughout as mol $\cdot$ min^{-1} $\cdot$ (ghost equivalent of the inside-out vesicle fraction)$^{-1}$ $\cdot 10^{15}$.

o-Phenanthroline/$CuSO_4$ treatment

To convert band 3 to disulfide cross-linked dimers, vesicles were treated immediately following homogenization with *o*-phenanthroline (250 μM) plus $CuSO_4$ (50 μM), final concentrations, in 5 mM sodium phosphate, pH 8, for 45 min on ice, followed by three washes in the buffer of interest [33]. Control vesicles showed no significant difference in kinetic parameters from untreated vesicles.

Results

Characteristics of the pyruvate transport system

The generation of inside-out vesicles occurs optimally in low ionic strength, alkaline media [26,27]. As shown in Table I, vesicle preparations enriched in inside-out species could be generated in the presence of 0.5 mM NADH, but at higher NADH levels, the yield dropped and contamination increased.

Does 0.5 mM NADH provide sufficient cofactor for the assay of pyruvate

TABLE I

THE EFFECT OF NADH CONCENTRATION ON VESICLE FORMATION

Vesicles were prepared in 0.5 mM sodium phosphate (pH 8) containing NADH, as outlined in Methods. The fraction of membranes present as sealed vesicles of each orientation was determined as previously reported [26,27]. Values are the results of five experiments ± the standard error of the mean.

NADH concentration (mM)	% Sealed vesicles	
	Inside-out	Right-side-out
0	37 ± 5	16 ± 2
0.5	36 ± 6	3 ± 3
1.0	25 ± 6	34 ± 10

flux? This level of cofactor is sufficient to saturate the enzyme until it is ⩾80% depleted, since the K_m of rabbit muscle lactate dehydrogenase for NADH under conditions comparable to these is less than 10^{-5} M [34]. Furthermore, the initial rate of the lactate dehydrogenase reaction in resealed ghosts was the same in the presence of 0.5 mM and 10 mM NADH. Finally, the time course observed using vesicles prepared with 0.5 mM NADH was linear for a significant period. We therefore assumed that the concentration of NADH did not affect the measurement of the initial rate of pyruvate flux into inside-out vesicles. However, the low levels did limit the extent of the transport process which could be measured.

In the vesicle system employed, the two principal rate-limiting steps in pyruvate reduction were its permeation through the membrane and its reaction with NADH via lactate dehydrogenase. Fig. 1 demonstrates that when an input of at least 100 units/ml was utilized, oxidation of NADH via lactate dehydrogenase was not rate-limiting. As with resealed ghosts [23], the large increase in the rate of reaction accompanying vesicle lysis with Triton X-100 provided additional evidence that the integrity of the membrane barrier controlled pyruvate reduction. In subsequent experiments, therefore, vesicles were prepared in 100 units/ml lactate dehydrogenase.

Figure 1 also demonstrates that, as in resealed ghosts [23], the rate of pyruvate flux into inside-out vesicles increased hyperbolically with pyruvate concentration in the external medium when the ionic strength was maintained constant at $\mu \geqslant 0.01$. (Ionic strength values are calculated from molar con-

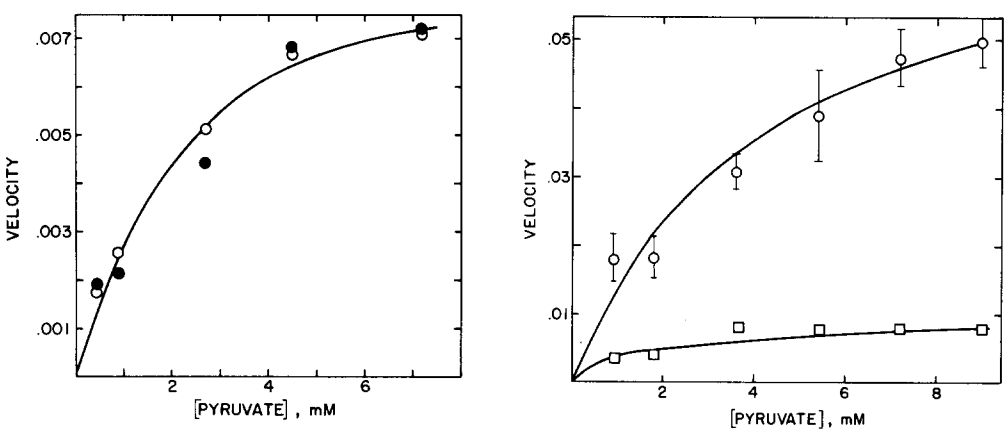

Fig. 1. Influence of sequestered lactate dehydrogenase on the determination of pyruvate flux. Vesicles were prepared in the presence of 0.5 mM NADH and lactate dehydrogenase at 100 (•) and 200 (○) units/ml. The assay was performed as described in Materials and Methods. The medium contained 80 mM sucrose; ammonium pyruvate and acetate concentrations were adjusted reciprocally to maintain a total ionic strength of 0.01 and pH 7.5. The assay temperature was 7° C. Velocities in this and subsequent figures are expressed as mol · min^{-1} · (ghost equivalent of the inside-out vesicle fraction)$^{-1}$ · 10^{15}.

Fig. 2. Pyruvate flux into resealed ghosts and inside-out vesicles. Membranes were sealed in the presence of 100 units/ml lactate dehydrogenase and 10 mM (ghosts) or 0.5 mM (vesicles) NADH, as described in Materials and Methods. The assay medium contained 80 mM sucrose; ammonium pyruvate and acetate were adjusted to a total ionic strength of 0.01 and pH 7.5. The assay temperature was 7° C. (○), resealed ghosts (error bars denote standard error of the mean); (□), inside-out vesicles.

centrations, assuming ideality.) The kinetic behavior of the pyruvate flux into inside-out vesicles differed characteristically from resealed ghosts under comparable experimental conditions (Fig. 2). For the vesicles, $K_t^C = 0.96 \pm 0.10$ mM and $V_t^C = 0.0089 \pm 0.0012$ ($n = 26$); for the ghosts, $K_t^E = 4.2 \pm 1.0$ mM and $V_t^E = 0.066 \pm 0.0096$ ($n = 10$) (cf. also ref. 23). This difference was not the result of the vesiculation step (e.g. an increased surface/volume ratio, smaller size or the elimination of traces of cytoplasmic constituents from the vesicles), since right-side-out vesicles exhibited kinetic parameters similar to resealed ghosts rather than inside-out vesicles (i.e. $K_t^E = 3.6 \pm 1.0$ mM and $V_t^E = 0.050 \pm 0.01$ under these conditions).

Effect of electrolytes on pyruvate transport

The dependence of the flux into inside-out vesicles on ammonium pyruvate concentration in the absence of other electrolytes was sigmoid (Fig. 3, control curve). In contrast, hyperbolic curves were found at a constant ionic strength of $\mu \geqslant 0.01$ (Figs. 1 and 2). Similar behavior has been observed for resealed ghosts [23,35].

Many electrolytes stimulated pyruvate flux into inside-out vesicles at constant substrate levels, as shown for sodium malate in Fig. 4. Ammonium salts gave identical results (not shown). Similar effects were observed in resealed ghosts, where it was shown that the stimulating electrolyte was only effective

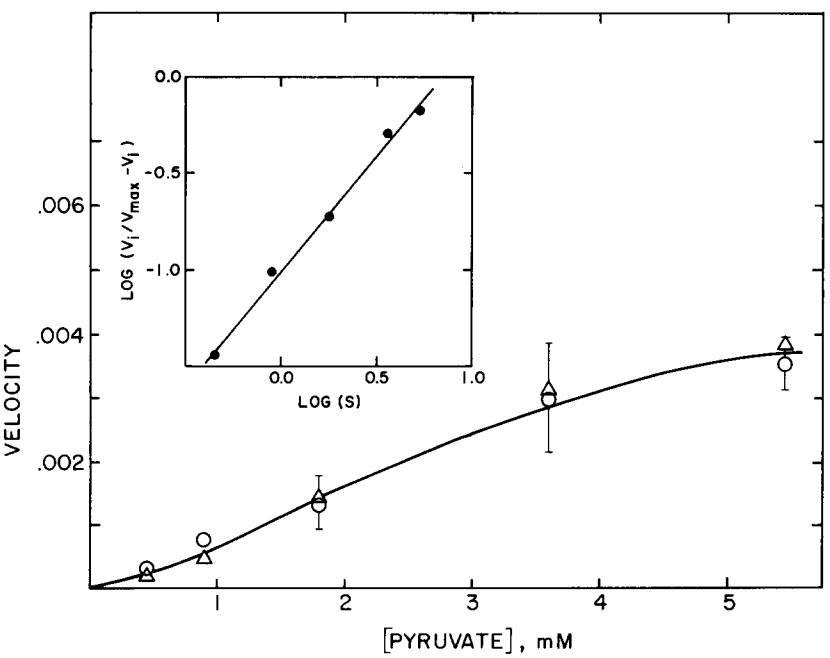

Fig. 3. Pyruvate flux into inside-out vesicles in the absence of other electrolytes: effect of papain digestion. Vesicles were prepared as described in Materials and Methods and incubated in the presence (△) or absence (○) of papain (1 μg/ml) on ice for 1 h. (The papain stock solution of 50 μg/ml was first activated by incubation in 5 mM DTT, 1 mM EDTA, as described in ref. 44.) The vesicles were then washed 3 times and the uptake of pyruvate assayed at 7° C in a medium containing 80 mM sucrose and ammonium pyruvate at pH 7.5. Inset: A Hill plot whose slope, the Hill coefficient, was $n_H = 1.2$.

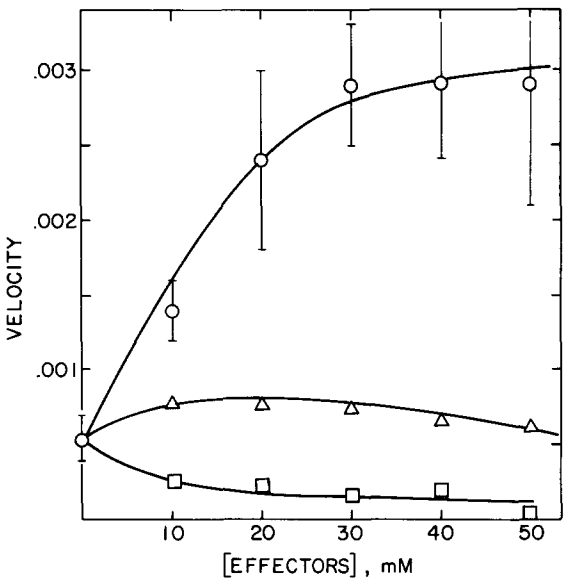

Fig. 4. The effect of electrolytes on pyruvate transport. Vesicles were prepared as described in Materials and Methods. The assay medium contained 80 mM sucrose, 0.9 mM ammonium pyruvate and the indicated concentrations of sodium salicylate (□), sodium chloride (△), and sodium malate (○). The assay pH was 7.6 and the temperature, 7° C. To eliminate transient light scattering artifacts caused by vesicle shrinkage, the membranes were pre-incubated on ice with an equal volume of double-strength electrolyte solution (minus pyruvate) just prior to assay.

in the external (i.e. pyruvate-containing) compartment [23]. To avoid this complexity, kinetic analyses were conducted at constant ionic strength, balancing ammonium pyruvate with ammonium acetate, as in Figs. 1 and 2.

Inhibitors of pyruvate influx

In contrast to the strong stimulation of pyruvate flux observed with salts of anions which are not carried by the anion transporter (e.g. malate and acetate; ref. 36), the transported anion, chloride [37—39], showed only a slight stimulation, reversed at high concentrations (Fig. 4). This effect was most simply explained by the competition of chloride with pyruvate for a common site.

Several compounds known to inhibit anion flux into erythrocytes [40,41] and pyruvate flux into resealed ghosts [23] also inhibited pyruvate entry into inside-out vesicles. These included the salicylate anion (Fig. 4), probenecid, 1-fluoro-2,4-dinitrobenzene and 4-acetamido-4′-isothiocyanato-2,2′-stilbene disulfonate (SITS) (Table II).

Probenecid instantly produced 90% inhibition of pyruvate flux into inside-out vesicles (Fig. 5B), but partially inhibited pyruvate flux in resealed ghosts only after a considerable lag (Fig. 5A). If probenecid acted only at the cytoplasmic surface, the delay in its effect on resealed ghosts could reflect the time required to cross the membrane. We found that this drug does, in fact, traverse the membrane, since erythrocytes were lysed by being suspended in isotonic ammonium probenecid solutions [23,36]. Probenecid probably crossed the membrane as the non-dissociated acid, in that the hemolysis time was not

TABLE II

EFFECT OF INHIBITORS OF PYRUVATE FLUX

Vesicles were prepared as described in Materials and Methods. The assay medium contained 80 mM sucrose; ammonium pyruvate and acetate were adjusted to a combined ionic strength of 0.01 at pH 7.5. The assay temperature was 7° C. Aliquots of vesicles were treated with one of three inhibitors as follows. Probenecid was added to the designated assay solution at 10 mM. Vesicles were treated with 10 mM 1-fluoro-2,4-dinitrobenzene for 30 min at 0° C, and washed twice in buffer before assay. Vesicles were treated with 1 mM SITS * for 90 min at 0° C, diluted 1 : 1 with 80 mM sucrose in 10 mM ammonium acetate, and used directly for assay.

Inhibitor	K_t^C (mM)	V_t^C (mol · min^{-1} · ghost^{-1}) · 10^{15}	% inhibition of V_t^C
None	0.96	0.0089	0
Probenecid	1.40	0.0062	30
1-fluoro-2,4-dinitro-benzene	1.40	0.0035	61
SITS *	1.11	0.0006	93

* 4-acetamido-4′-isothiocyanato-2,2′-stilbene disulfonate.

prolonged by the addition of a potent inhibitor of anion transport, 0.1 mM SITS [35]. These finding suggest that the site of probenecid action is at the cytoplasmic surface.

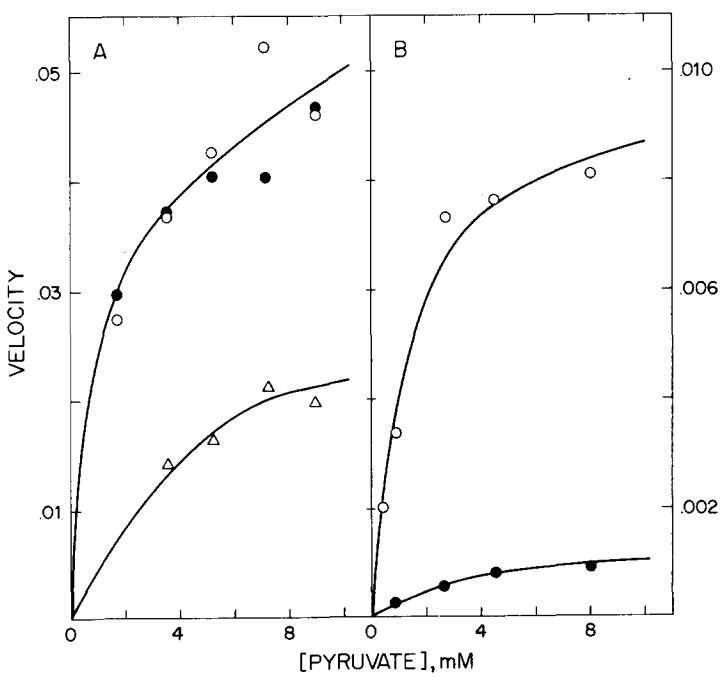

Fig. 5. Probenecid inhibition of pyruvate transport. Membranes were prepared as described in Materials and Methods. The assay medium contained 80 mM sucrose; ammonium pyruvate and acetate were adjusted to a combined ionic strength of 0.01 and pH 7.4. The assay temperature was 7° C. (A) Resealed ghosts. The assay medium contained 0 (○) or 10 mM ammonium probenecid (●,△). One set of samples (●), recieved probenecid at zero time (i.e. at the initiation of assay) while the other samples (△) were preincubated with probenecid for 30 min at 0° C prior to assay. (B) Vesicles. The assay medium contained 0 (○) or 10 mM probenecid (●), added at zero time (the initiation of the assay).

The effect of specific ligands of band 3 on pyruvate flux

There is strong evidence, albeit indirect, that band 3, the predominant membrane-spanning polypeptide in the human erythrocyte membrane, is the site of anion transport [16—18]. We therefore tested whether aldolase [42] or glyceraldehyde 3-P dehydrogenase [43], both of which bind specifically to the cytoplasmic pole of band 3, affect pyruvate flux. Inside-out vesicles were incubated in the presence of saturating amounts of these enzymes, washed, and pyruvate flux analyzed. (Polyacrylamide gel electrophoresis in sodium dodecyl-sulfate verified the binding of the enzymes to the membranes.) No statistically significant differences were observed between vesicles saturated with either enzyme and those having no bound enzyme. Further experiments in which the glycolytic enzymes were present at 1 mg/ml in the assay medium itself gave the same results.

The effect of papain digestion on pyruvate flux

Papain digestion of the exposed surface of inside-out vesicles cleaves an approx. 40 000-dalton cytoplasmic domain from the approx. 90 000-dalton band 3 polypeptide [44]. The 40 000-dalton domain, which bears binding sites for glyceraldehyde-3-phosphate dehydrogenase and aldolase [43,44], is released into solution by this digestion, while the complementary approx. 50 000-dalton piece remains membrane-bound [44].

We digested inside-out vesicles with papain and verified by polyacrylamide gel electrophoresis that the cleavage of band 3 was complete. As shown in Fig. 3, this treatment caused no significant alteration of pyruvate flux. (Similar negative results were obtained when the assay was performed at μ = 0.01, whether in the presence of ammonium acetate, glucuronate or chloride; ref. 35.) Furthermore, trypsin digestion, which leads to the excision of the same region of the band 3 polypeptide, caused no significant change in pyruvate flux into vesicles.

Since the action of probebecid appears to be confined to the cytoplasmic surface of the red cell membrane (see above), we studied the effect of excising the cytoplasmic pole of band 3 on probenecid inhibition of pyruvate flux. Under conditions where $\geqslant$95% of the band 3 molecules present were cleaved, probenecid still inhibited V_t^C by ~90%. The site of probenecid action must therefore not reside in the approx. 40 000-dalton cytoplasmic-surface domain of band 3.

Finally, proteolytic cleavage had no effect on ionic activation curves of the type depicted in Fig. 4 for sodium malate.

The effect of o-phenanthroline/$CuSO_4$ treatment on pyruvate flux

This chelate complex catalyzes the air oxidation of sulfhydryls to disulfides, inducing a cross-link between band 3 polypeptides at their cytoplasmic pole to form covalent dimers [33]. Inside-out vesicles cross-linked as described in Materials and Methods showed a diminished pyruvate influx, caused by an increase in their K_t^C (at μ = 0.01 and 7° C) from 0.96 ± 0.10 to 2.1 ± 0.10 mM, without a change in V_t^C. This effect was reversed by incubation of the vesicles with 100 mM dithiothreitol, which reduces the band 3 cross-link induced by *o*-phenanthroline/$CuSO_4$ [33], and by digestion with papain or trypsin, which

excises the cross-linked cytoplasmic domain of band 3 [44].

Cross-linking did not alter the inhibition by probenecid of pyruvate flux into inverted vesicles.

In contrast to inside-out vesicles, ghosts treated with o-phenanthroline plus $CuSO_4$ prior to resealing showed an enhanced pyruvate influx under these assay conditions, apparently the result of an increase in both K_t^E and V_t^E [35].

Discussion

We have adapted a technique previously described for resealed ghosts [23] the examination of pyruvate flux into inside-out vesicles, so as to analyze the transport of this anion from both membrane surfaces under defined conditions. An important limitation was encountered. Since endocytic vesiculation occurs optimally at $\mu \sim 0.003$, we were unable to incorporate high concentrations of electrolytes within these vesicles. One result of this was the modest amount of internal NADH attainable (see Table I); thus, neither long time courses nor very rapid fluxes could be monitored. A more serious consequence was the exclusion of high levels of transportable anions from the inner compartment. Other studies [45,46] have indicated that this transporter conducts primarily 1 : 1 anion exchange, rather than net flux. Thus, pyruvate transport will be influenced by contralateral anions, which could become rate-limiting at low concentrations [35].

Three permeant anions species were available for exchange with pyruvate in these vesicles. Phosphate (albeit 0.5 mM) is the buffer in which the membranes were sealed. Furthermore, the lactate dehydrogenase-catalyzed reaction generates not only lactate but, by consuming protons, releases hydroxyl anions as well:

$$(\text{Pyruvate})^- + \text{NADH} + H_2O \rightarrow (\text{lactate})^- + \text{NAD}^+ + OH^- \quad (1)$$

(Traces of the ubiquitous bicarbonate ion could also mediate the flux of hydroxide via the anion transporter [38].) Preliminary studies indicate that under minimally-buffered conditions such as these, OH^- (rather than lactate) is released from vesicles in stoichiometric proportion to the pyruvate taken up [35].

In the absence of a full characterization of the impact of contralateral hetero-anions on anion transport, the kinetic parameters reported here (and the asymmetry they imply) cannot be unambiguously interpreted as intrinsic functions of this membrane. It should be noted, however, that both the resealed ghosts and inverted vesicles had comparable, low concentrations of internal anions (see Fig. 2). Furthermore, right-side-out vesicles generated in a medium very similar to inside-out vesicles had the kinetic properties of resealed ghosts and not inverted vesicles (see Results).

Many similarities between the resealed ghost [23] and inside-out vesicle systems were observed. (a) The dependence of velocity on the concentration of the pyruvate salt was sigmoid in both systems in the absence of other electrolytes. This effect appears to derive from the strong influence of ionic strength of the external medium on flux rate. Our recent studies have indicated that this stimulation is a function of the cation present, since Ca^{2+} and Ba^{2+} are far more

potent than Na^+, NH_4^+ or $(Tris)^+$ at a given ionic strength. The action of the cation appears not to be through screening of the membrane's repulsive anionic charges, since it is V_t which is stimulated rather than K_t, as a charge screening effect would require. In fact, raising ionic strength actually increases K_t [23,35]. (b) Salts of non-transported electrolytes (such as malate and acetate) stimulated pyruvate flux greatly (Fig. 4) while salts of transported anions (e.g. chloride) had a superimposed inhibitory effect, presumably competitive. (c) Certain non-competitive inhibitors of pyruvate flux, such as 4-acetamido-4′-isothiocyanato-2,2′-stilbene disulfonate (SITS) and 1-fluoro-2,4-dinitrobenzene, were effective at both sides of the membrane (Table II). Since SITS does not cross the membrane under these experimental conditions [15], it may be that it can inhibit anion transport by interacting with either surface. Similarly, Zaki et al. [17] have demonstrated that certain sulfonic acid derivatives inhibit sulfate transport non-covalently when present at either surface of resealed ghosts.

Significant differences between the two systems were also noted. (a) Kinetic parameters obtained in right-side-out and inside-out systems under comparable conditions differed characteristically (see Fig. 2 and above discussion). (b) Two modes of asymmetry were observed in the effect of *o*-phenanthroline/$CuSO_4$ cross-linking. First, this chelate complex was effective only when applied to the cytoplasmic surface [23], at which it cross-links band 3 [44]. Secondly, pyruvate flux increased in resealed ghosts [35] but decreased inside-out vesicles following cross-linking. (c) Probenecid, which appears to cross the membrane as the undissociated acid, appeared to act only at the cytoplasmic surface, although it inhibited flux in both directions (Fig. 5).

Binding glyceraldehyde 3-P dehydrogenase and aldolase to band 3 had no effect on pyruvate flux in either inside-out vesicles or resealed ghosts [35]. A related finding has been reported for glucose transport [47]. This result is consistent with the fact that excision of the binding site for these enzymes by papain or trypsin caused no significant change in transport kinetics (Fig. 3). Probenecid inhibition was also unchanged by digestion of inside-out vesicles, although its site of action is presumably at the cytoplasmic surface. On the other hand, *o*-phenanthroline/$CuSO_4$ treatment, which induces disulfide cross-linking of this domain in band 3 [33,44], did affect transport kinetics in both directions. These data suggest that the 40 000-dalton cytoplasmic pole of band 3 may influence pyruvate transport without being an essential component. Of course, these interpretations are contingent on the premise that band 3 is the anion transporter.

Recently, Halestrap [48,49] observed two kinetically-distinguishable pyruvate transport systems in intact human erythrocytes. A high K_t system was found to be inhibitable by chloride ions; the other, low K_t system was detected only in red cells containing high levels of lactate. Under our experimental conditions, only the former system, the common anion transporter, seemed to contribute to pyruvate flux kinetics. That is, pyruvate appeared to exchange for OH^- and not lactate. Furthermore, pyruvate flux was inhibited here by covalent stilbene disulfonates and by anions such as chloride, as befits the pyruvate/Cl^- exchange system of Halestrap [48,49]. Since we have previously shown that the K_t for pyruvate uptake into resealed ghosts rises sharply with

ionic strength [23], the low K_t values reported here may not be inconsistent with that of Halestrap, given the difference between the electrolyte levels in the two studies.

Acknowledgments

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