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KINETICS OF NUCLEOSIDE TRANSPORT IN HUMAN ERYTHROCYTES

ALTERATIONS DURING BLOOD PRESERVATION

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The transmembrane equilibration of radiolabeled uridine was measured by rapid kinetic techniques in human erythrocytes from freshly drawn blood and in the same cells during conventional storage of the blood as well as in cells from outdated blood. Our results confirm earlier reports that the maximum velocity of uridine equilibrium exchange (V^{ee}) at 25°C is about 30% lower in outdated than fresh red cells, whereas the opposite is the case for the Michaelis-Menten constant for equilibrium exchange (K^{ee}), and that maximum zero-*trans* efflux (V_{21}^{zt}) is about 4-times greater than maximum zero-*trans* influx (V_{12}^{zt}) in outdated cells (directional asymmetry), whereas they are about the same in fresh red cells. At 25°C, the nucleoside-loaded carrier of fresh cells moves on the average 6-times more rapidly than the empty carrier, whereas the differential mobility of loaded and empty carrier from outdated cells is about 15-fold. Our results also show that greater efflux than influx in outdated cells is not due to a general leakiness of outdated cells, that the differences in kinetic properties of the transporter developed during the first two weeks of blood storage and that the differences are greatly amplified when transport is measured at 5°C rather than 25°C. At 5°C, the loaded carrier from outdated red cells moves about 325-times more rapidly than the empty carrier and maximum zero-*trans* efflux exceeds maximum zero-*trans* influx about 14-times, whereas the transport of fresh cells exhibits directional symmetry just as at 25°C. The changes in kinetic properties of transport induced by temperature and storage are probably related to structural alterations in the plasma membrane and suggest that the operation of carrier is subject to modification by the membrane environment. Other results show that the kinetics of the sugar transport of human red cells is not affected in the same manner by blood storage as those of the nucleoside transporter.

Introduction

There is general agreement that nucleoside transport across the membrane of human erythrocytes [1–4] and cultured mammalian cells [3] is adequately described by the simple carrier model [5]. Results from our laboratory indicated that uridine transport in both erythrocytes and cultured cells is indifferent with respect to direction (directional symmetry), but that the substrate-loaded carrier moves ** 3–8-times more

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** 'Mobility' connotes some macromolecular movement, if only a conformational shift.

*** As defined by Stein [5], 'zero-*trans*' designates the transport of a substrate from one side of the membrane (the *cis* side) to the other side, where its concentration is zero. 'Equilibrium exchange' designates unidirectional flux of radioactively labeled substrate from one side to the other side of the membrane, where substrate is held at equal concentration. Arbitrarily, we designate the outside and inside faces of the membranes as 1 and 2, respectively.

rapidly than the empty carrier in erythrocytes, whereas the mobility of the transporter of cultured cells is equal whether loaded or empty. Differential mobility of loaded and empty carrier in red cells was suggested by the earlier finding [6] that the rate of nucleoside movement across the membrane is stimulated by the presence of excess substrate on the *trans* side of the membrane ('*trans*-stimulation'), but interpretation of such results is not unambiguous [3]. Unequivocal evidence for differential mobility of loaded and empty carrier, on the other hand, is a higher maximum velocity of equilibrium exchange *** (V^{ee}) than of zero-*trans**** entry or exit (V_{12}^{zt} and V_{21}^{zt}, respectively) coupled with equality of the first-order rate constants for equilibrium exchange and zero-*trans* flux ($V^{ee}/K^{ee} = V^{zt}/K^{zt}$) as stipulated by the simple carrier model (see Table I and Ref. 3). Directional symmetry is recognized by $V_{12}^{zt} = V_{21}^{zt}$, i.e., the maximum velocity of zero-*trans* entry is equal to maximum velocity of exit.

Our results with human red cells differed from those reported earlier by Cabantchik and Ginsburg [1] who reported that V_{21}^{zt} was about 4-times higher than V_{12}^{zt} (directional asymmetry, Table I). This discrepancy has been reconciled recently by Jarvis et al. [4] on the basis of differences in kinetic properties of uridine transport in red cells from outdated blood, as used by Cabantchik and Ginsburg [1], and from fresh blood, as used by ourselves (see Table I), in that the transporter of cells from freshly drawn blood exhibits directional symmetry, whereas that of cells from outdated blood exhibits directional asymmetry. The reason for this difference in kinetic properties is not known, but it may reflect some change in cell membrane structure which causes a lowering in the outward mobility of empty carrier. In this regard it is of interest that the fluidity of the red-cell membrane has been reported to increase during storage, particularly as a result of decreases in ATP concentration [7]. The suggestion is that this change may affect the conformation of integral membrane proteins. It is well known that the maintenance of appropriate concentrations of ATP in red cells is very important in preserving the integrity of the membrane [8].

We have conducted additional experiments designed to characterize more fully the red-cell transport changes that occur during blood preservation. First, we rigorously determined the kinetics of transport in cells from several batches of fresh and outdated blood by integrated rate analysis of complete or extended time-courses of substrate equilibration. Second, we have followed erythrocytes during storage to assess when changes in nucleoside transport occurred, and have inquired into the molecular basis of these changes.

Experimental Procedures

Fresh human erythrocytes were a by-product of lymphocyte isolation from blood of normal human volunteers, and were kindly supplied by R.D. Estensen, and J. Kersey, Department of Pathology, University of Minnesota. Outdated human whole blood or red cells supplemented with a conventional solution of citrate, phosphate, dextrose and adenine (CPDA-1) were obtained from the blood bank, University of Minnesota Hospitals, and used within 3 weeks after the expiration date. In one experiment 500 ml fresh whole human blood in CPDA-1 (LP) was obtained through the Blood Donor Center, University of Minnesota, and monitored during storage. All blood was stored at 4°C. For experiments, the erythrocytes were washed three or four times in cold saline buffered with 5 mM Tris-HCl (pH 7.4: Tris-saline) and suspended in the same solution.

Detailed time-courses of accumulation of [5-^{3}H]uridine (Moravek Biochemicals, Brea, CA) by cells or its release from preloaded cells were determined by a rapid kinetic technique both under the zero-*trans* and equilibrium exchange conditions as described previously [2,3] or the suspensions were sampled by manual sampling of cell substrate mixtures where indicated in appropriate experiments. In either approach the cells were rapidly separated from the medium by centrifugation through an oil mixture [9] and analyzed for radioactivity. Radioactivity in cell pellets was corrected for that trapped in extracellular water space (about 10% of total water space in cell pellets) and converted to pmol substrate/μl cell water on the basis of experimentally determined [9] cell water spaces. The following equation was fitted to the zero-*trans* entry time courses under the conditions

stated in individual experiments:

$$S_{2,t} = S_1\left[1-\exp\left(-\frac{t+(R_{21}+R^{ee}S_1/K)S_{2,t}}{KR_{oo}+R_{12}S_1-R_{21}S_1+S_1{}^2R^{ee}/K}\right)\right] \quad (1)$$

where $S_{2,t}$ = concentration of intracellular substrate at time t ($S_{2,0}=0$); S_1 = extracellular substrate concentration (taken as a constant); K is a limit Michaelis-Menten constant (intrinsic dissociation constant, Ref. 5); and the R terms are resistance factors, proportional to the round-trip time of the carrier in one of four modes [3,5].

Analysis of zero-*trans* exit and of inward equilibrium exchange data are described along with individual experiments. Equations were fitted by a non-linear least squares regression program based on the algorithm of Dietrich and Rothmann [10]. Kinetic parameters are stated ± S.E. of the estimate. Equilibrium binding of nitrobenzylthioinosine (NBTI) was measured as described previously [11].

Results and Discussion

Blood (LP) was collected by the Blood Collection Center and the fresh red cells were assayed the same day at 25 °C for transmembrane equilibration of 5 mM uridine in the inward and outward equilibrium exchange and zero-*trans* entry modes. The blood was stored at 4 °C and at weekly intervals cells were removed and assayed in the same manner. The use of a very high concentration of uridine assured that transport was saturated in both fresh and outdated cells, so that the initial velocities approximated maximum velocities.

The results for cells from fresh blood and from the same blood after 7 weeks of storage are illustrated in Fig. 1A and B, respectively. Clearly the initial velocities of zero-*trans* entry (v_{12}^{zt}) were markedly lower in cells from 7-week-old blood than in cells from fresh blood, whereas equilibrium exchange flux was reduced only about 20%. Fig. 1C illustrates that the decrease in v_{12}^{zt} occurred rapidly during the first 2 weeks of storage of the

TABLE I

PREVIOUSLY REPORTED KINETIC PARAMETERS FOR URIDINE TRANSPORT IN HUMAN RED CELLS FROM FRESHLY DRAWN AND OUTDATED BLOOD

Experimental Protocol	Parameters	Erythrocytes			
		Outdated [a] (25 °C)	Fresh [b] (25 °C)	Outdated [c] (22 °C)	Fresh [c] (22 °C)
Zero-*trans* influx	K_{12}^{zt} (μM)	73	120	130	170
	V_{12}^{zt} (μM/s)	9	41	10	32
	V_{12}^{zt}/K_{12}^{zt} (s^{-1})	0.12	0.34	0.078	0.19
Zero-*trans* efflux	K_{21}^{zt} (μM)	400	120	380	140
	V_{21}^{zt} (μM/s)	33	41	33	37
	K_{21}^{zt}/K_{12}^{zt} (s^{-1})	0.082	0.34	0.087	0.26
Inward equilibrium exchange	K^{ee} (μM)	1280	540	1100	760
	V^{ee} (μM/s)	120	148	98	128
	V^{ee}/K^{ee} (s^{-1})	0.094	0.27	0.089	0.17
	R_{00}/R^{ee}	16	6.1	12	6.4

[a] Data from Cabantchik and Ginsburg [1], converted to uniform units.
[b] Data from Plagemann and Wohlhueter [2].
[c] Data from Jarvis et al. [4], converted to uniform units.

blood under routine preservation conditions (citrate, phosphate, dextrose, adenine). The maximum decrease in v_{12}^{zt} had occurred after 2 weeks of storage, i.e., long before the accepted normal expiration data of the blood (6 weeks). Entry of 5 mM uridine under zero-*trans* conditions was extremely slow in outdated cells (Fig. 1B). The question, therefore, arose of whether transmembrane equilibrium is eventually attained as expected in the operation of a simple carrier. This was the case, but transmembrane equilibration of 5 mM uridine was approached only after about 2 h at 25 °C (data not shown), whereas in equilibrium exchange the radiolabeled substrate equilibrated in only a few min in outdated cells (Fig. 1B), just as in fresh cells (Fig. 1A).

We measured under comparable experimental conditions the inward equilibrium exchange of uridine at 25 °C over a wide range of concentrations by our rapid kinetic technique. We found K^{ee} to be slightly higher for outdated than for fresh red cells, whereas the reverse was the case for V^{ee} (Table II). The combined increase in K^{ee} and decrease in V^{ee} indicates, however, that in the first-order concentration range uridine equilibrium exchange at 25 °C was only about one half as rapid in outdated than in fresh red cells (see V^{ee}/K^{ee} ratios). These results are in agreement with the combined data from the earlier studies by Cabantchik and Ginsburg [1], ourselves [2] and Jarvis et al. [4] (Table I). A complete kinetic comparison of uridine transport in fresh and outdated red cells, however, required measurements of zero-*trans* influx and efflux over wide ranges of uridine concentrations. Technical difficulties made it impossible to obtain accurate values for uridine efflux at 20–25 °C over the appropriate substrate concentration ranges. Our rapid kinetic methodology employing a dual-syringe apparatus as we have previously applied for estimating zero-*trans* efflux [2] is not strictly applicable because of the presence of a finite concentration of substrate on the *trans* side (1/8.31 of that on the *cis* side, Ref. 2) at the start of measuring $S_{2,t}$ (i.e. at $t = 0$). To

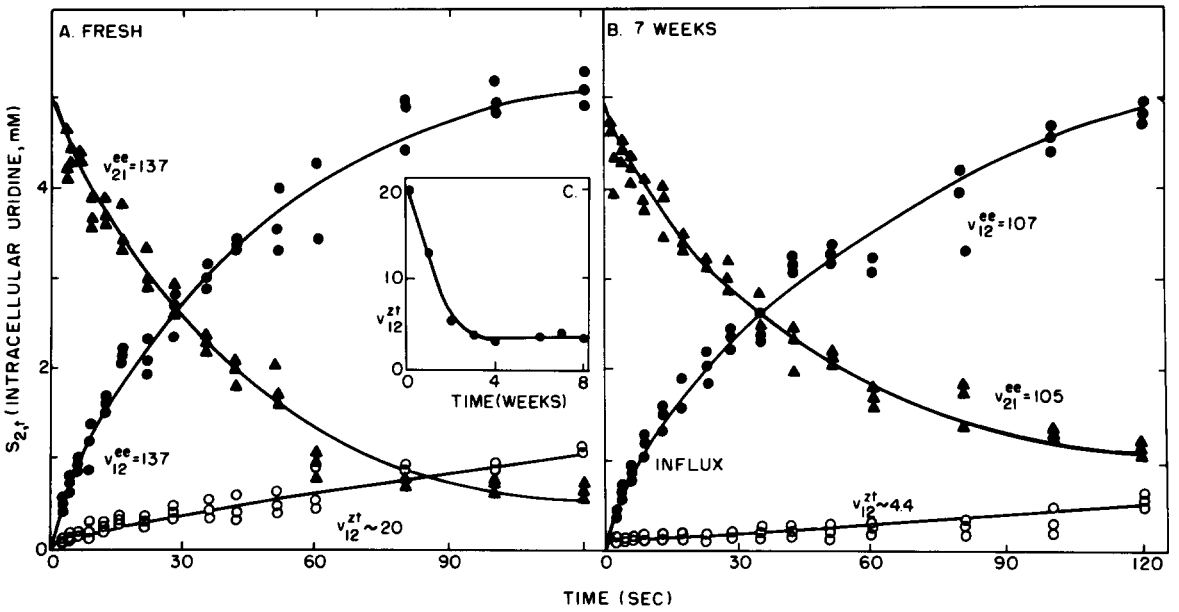

Fig. 1. Zero-*trans* influx and inward and outward equilibrium exchange of 5 mM uridine in red cells from freshly drawn blood and from the same blood at weekly intervals of storage at 4 °C. The blood was supplemented with citrate, phosphate, D-glucose and adenine during collection. Red cells were collected by centrifugation, washed three times in Tris-saline and then suspended in the same to about $6 \cdot 10^8$ cells/ml. Time courses of transmembrane equilibration of 5 mM [^{3}H]uridine at 25 °C were measured in triplicate by rapid kinetic techniques using a dual-syringe technique as described in Experimental Procedures. Detailed results for fresh and 7-week-old red cells are presented in frames A and B, respectively. Frame C summarizes the rates of zero-*trans* entry of the cells as a function of time of storage at 4 °C.

TABLE II

KINETIC PARAMETERS FOR EQUILIBRIUM EXCHANGE AT 25 °C AND 5 °C BY HUMAN ERYTHROCYTES FROM FRESHLY DRAWN AND OUTDATED BLOOD

Inward equilibrium exchange of 40, 80, 160, 320, 640, 1280, 2560 and 5120 μM [^{3}H]uridine (between 300 and 500 cpm/μl, irrespective of concentration in different experiments) was measured by rapid kinetic techniques as described under Experimental Procedures. The final cell densities were about $5 \cdot 10^8$ cells ml. The best-fitting parameters ± S.E. of estimate were obtained by integrated rate analysis [2]. The following equation was fitted to the data for the eight uridine concentration: $N_{2,t} = N_{2,\infty}[1 - \exp(V^{ee}t/(K^{ee} + S)]$ where $N_{2,t}$ = intracellular concentration of radiolabel at time t; $N_{2,\infty}$ = the equilibrium concentration of radioactivity in the cells, which was equal to N_1, the radioactivity in an equivalent volume of medium; S = chemical concentration of substrate; and K^{ee} and V^{ee} are the apparent Michaelis-Menten constant and maximum velocity for equilibrium exchange, respectively. Data in first row are from Plagemann and Wohlhueter [12]. All other values represent independent kinetic experiments with different batches of red cells not previously reported.

Erythrocytes	25 °C			5 °C		
	K^{ee} (μM)	V^{ee} (μM/s)	V^{ee}/K^{ee} (s^{-1})	K^{ee} (μM)	V^{ee} (μM/s)	V^{ee}/K^{ee} (s^{-1})
Fresh	805 ± 61	172 ± 4	0.46	279 ± 23	10.4 ± 0.38	0.037
	507 ± 173	234 ± 16	0.21	323 ± 16	7.7 ± 0.12	0.024
Outdated	1090 ± 136	154 ± 5	0.14	1336 ± 110	4.6 ± 0.20	0.0034
	1036 ± 100	132 ± 3	0.13	628 ± 47	3.5 ± 0.11	0.0056
				1445 133	5.5 ± 0.28	0.0038

obtain true zero-*trans* efflux data, the cells need to be preloaded to equilibrium with substrate, then collected by centrifugation for removal of extracellular substrate and resuspended in fresh medium devoid of substrate (zero-*trans*). One then measures the appearance of substrate in the extracellular fluid or the loss of substrate from the cells or both. In our hands, the loss of uridine was so fast at 25 °C (at low uridine concentrations) that most of it had already been released from the cells after we had resuspended the cells and taken the first sample (15 s), so that it was impossible to estimate accurate initial exit velocities. A typical analysis comparing zero-*trans* entry and exit of 400 μM uridine in cells from fresh and outdated blood is shown in Fig. 2A and illustrates the problem. Only at concentrations well above the presumed Michaelis-Menten constant (i.e., at ⩾ 3.2 mM, Fig. 2B) was release of radioactivity from the cells slow enough to allow reasonably valid estimates of rates of release. These data and similar data from at least four other experiments of this type clearly showed, however, that efflux was also reduced in outdated cells (30–50%), but to a lesser extent than influx. Since at these high uridine concentrations the initial velocity of exit approaches the maximum velocity (V_{21}^{zt}), our results indicate that V_{21}^{zt} is lower for outdated than fresh red cells, which is contrary to earlier reports (see Table I). Nevertheless, all data support the conclusion that efflux is more rapid than influx in outdated red cells. One experimental artifact which could account for this finding and which had not been considered in earlier work was that apparent higher efflux than influx was due to a general leakiness of the membrane of outdated cells. To assess this possibility, we have compared the uptake of radiolabeled L-glucose by outdated and fresh red cells and its release from these cells (Fig. 3). The finding that both were the same in fresh and outdated cells rules out an increased leakiness of cells as the cause of more rapid efflux than influx of uridine in outdated cells.

For a kinetic comparison of zero-*trans* influx and efflux, we have turned to measurements at 5 °C, because in other experiments [12] we have demonstrated that at this temperature efflux is slow enough to be measured by manual sampling. Red cells were preloaded (37 °C), at a 50% suspension, with various concentrations of [^{3}H]uridine. Then the suspension was diluted 15-fold with ice-cold Tris-saline and the cells were rapidly collected by centrifugation and suspended to 10% in Tris-saline at 5 °C. At various times of incubation at

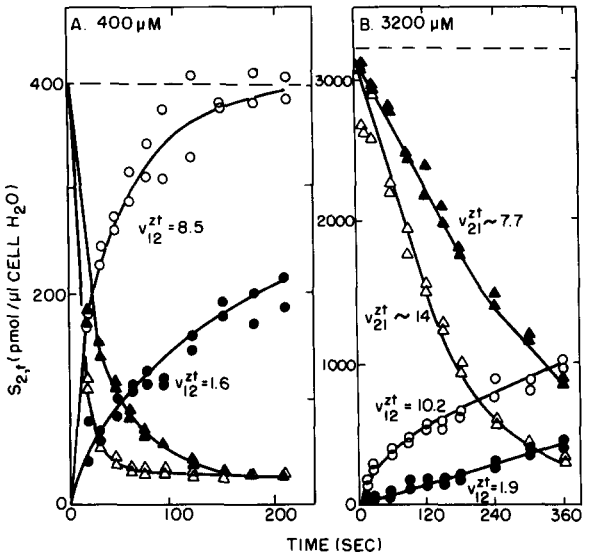

Fig. 2. Zero-*trans* influx (○———○, ●———●) of efflux (△———△, ▲———▲) of 400 (A) and 3200 (B) μM [^{3}H]uridine by fresh (open symbols) and outdated red cells (solid symbols). Portions of suspensions of $2.5 \cdot 10^9$ washed, fresh or outdated erythrocytes per ml were supplemented with 400 (A) or 3200 μM (B) [^{3}H]uridine (1500 cpm/μl, irrespective of concentration; zero-*trans* efflux) or remained without addition (zero-*trans* influx). After 60 min of incubation at 37 °C, the suspensions were cooled to 0 °C, rapidly diluted 15-fold with ice-cold Tris-saline, the cells were collected by centrifugation at 4 °C and suspended to $5 \cdot 10^8$ cells/ml in Tris-saline (zero-*trans* efflux) or Tris-saline containing 400 (A) or 3200 μM (B) [^{3}H]uridine (300 cpm/μl, irrespective of concentration; zero-*trans* influx) all equilibrated at 25 °C. At the indicated times of incubation at 25 °C, the cells from duplicate 0.5 ml samples of suspension were collected by centrifugation through oil and analyzed for radioactivity. Radioactivity values were corrected for that trapped in extracellular space and converted to pmol/μl cell water on the basis of the cellular water space estimated on separate samples of the same cell suspension. Equation 1 was fitted to the zero-*trans* entry time-courses with R^{ee} and K fixed at values independently determined for fresh and outdated cells (see Table II and Ref. 2). v_{12}^{zt} was calculated as $S_1 V_{12}^{zt}/(K + S_1)$. v_{21}^{zt} values were estimated graphically, where possible, from the linear portions of the exit curves.

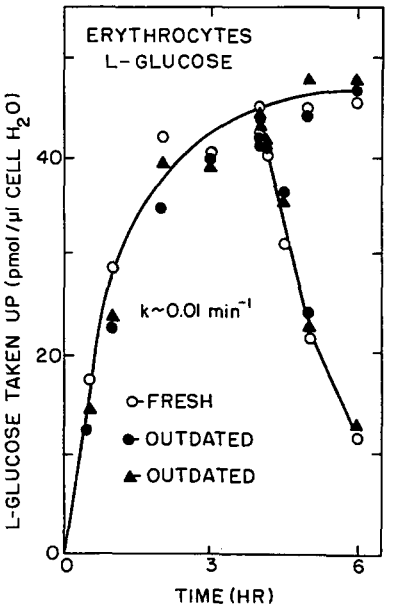

Fig. 3. L-Glucose uptake and release in fresh and outdated red cells. Suspensions of washed red cells were supplemented with 50 μM L-[^{3}H]glucose and incubated at 25 °C. At the indicated times, the cells from duplicate samples were collected by centrifugation and analyzed for radioactivity. At 4 h of incubation, the cells from a portion of each cell suspension were collected by centrifugation, and resuspended in fresh medium. Incubation and sampling were continued. All points are averages of the duplicate samples. Results are for cells from two different batches of outdated blood.

5 °C, the cells from duplicate 0.5 ml samples of suspension were collected by centrifugation through oil and analyzed for radioactivity. Representative time-courses of zero-*trans* uridine release from outdated cells are illustrated in Figs. 4D–F from which exit velocities were estimated by linear regression. A fit of the Michaelis-Menten equation to the velocities estimated for seven uridine concentrations yielded a $K_{21}^{zt} = 88 \pm 22$ μM and a V_{21}^{zt} $(1/R_{21}) = 0.223 \pm 0.022$ pmol/μl cell water per s (Fig. 5).

For estimating K_{12}^{zt} and V_{12}^{zt}, we determined the time courses of intracellular accumulation of radiolabeled uridine at various concentrations under zero-*trans* conditions at 5 °C. Representative time-courses are illustrated in Figs. 4A–C. Then the zero-*trans* influx equation was fitted to the zero-*trans* entry data with R_{21} $(= 1/V_{21}^{zt})$ and R^{ee} $(1/V^{ee})$ fixed at the values determined experimentally (see Fig. 5 and Table II, respectively). The fit yields estimates of K, the limit Michaelis-Menten constant, and R_{12} $(= 1/V_{12}^{zt})$ from which K_{12}^{zt} and R_{00} were calculated as well as independent estimates of K_{21}^{zt} and K^{ee} which are summarized in Table III. The finding that these latter estimates were similar to those estimated directly from the zero-*trans* efflux and the equilibrium exchange

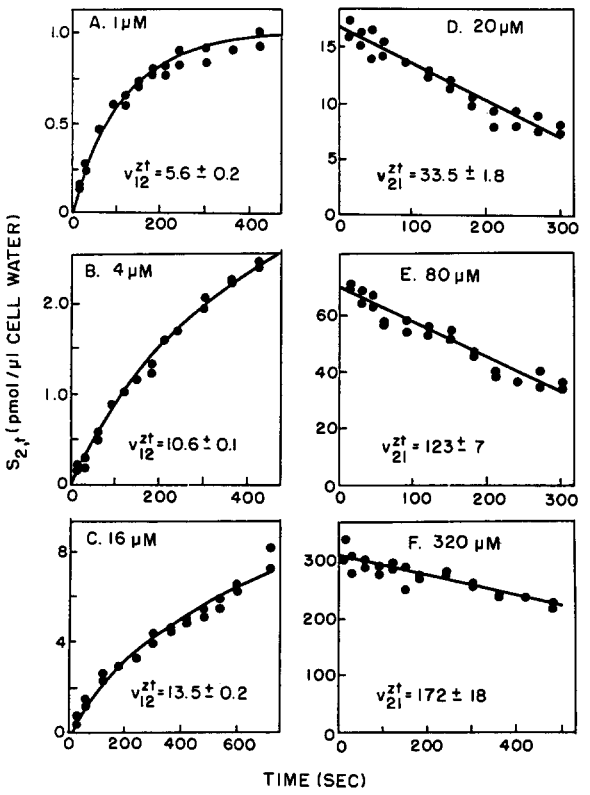

Fig. 4. Kinetic analysis of zero-*trans* influx and efflux in outdated red cells at 5 °C. The experiment was conducted basically as described in the legend to Fig. 2, except that after preincubation the cells were suspended to $5 \cdot 10^8$ cells/ml in medium that was equilibrated at 5 °C, and the suspensions were manually sampled during incubation at the same temperature. The concentrations of uridine were 0.5, 1, 2, 4, 8, 16 and 32 μM for influx and 10, 20, 40, 80, 160, 320 and 640 μM for efflux. Representative time courses of zero-*trans* entry and exit are illustrated in frames A–C and D–F, respectively. v_{21}^{zt} was computed by linear regression of the zero-*trans* exit time-courses and K_{21}^{zt} and V_{21}^{zt} were obtained by fitting the Michaelis-Menten equation to the values (see Fig. 5). Equation 1 was fitted to the zero-*trans* entry time-courses pooled for the seven uridine concentrations with R^{ee} fixed at 0.22 s/μM (see Table II) and R_{21} ($= 1/V_{21}^{zt}$) at 4.5 s/μM (see Fig. 5) which yielded best fitting values for R_{12} and K, which are stated in Table III.

data, respectively, and that the first-order rate constants (maximum velocity/Michaelis-Menten constant) were similar for the three experimental protocols (Fig. 5, Table II) indicate that the simple carrier model adequately describes uridine transport at 5 °C in outdated red cells, in spite of the

TABLE III

KINETIC PARAMETERS FOR ZERO-*TRANS* INFLUX AT 5 °C IN OUTDATED HUMAN ERYTHROCYTES

The experimental details are described in the text and in the legend to Fig. 5. The indented parameters were calculated directly from the fit parameters K and R_{12}: $R_{00} = R_{12} + R_{21} - R^{ee}$; $V_{12}^{zt} = 1/R_{12}$; $K_{12}^{zt} = KR_{00}/R_{12}$; $K_{21}^{zt} = KR_{00}/R_{21}$; and $K^{ee} = KR_{00}/R^{ee}$. For comparison, previously reported data for fresh erythrocytes [12] are also presented.

Parameters	Erythrocytes	
	Outdated	Fresh
K (μM)	1.5 ± 0.1	2.8 ± 0.3
R_{12} (s/μM)	67 ± 1	1.6 ± 0.04 ($= R_{21}$)
$r_{y\hat{y}}$	0.976	0.983
K_{12}^{zt} (μM)	1.6	5.4 ($= K_{21}^{zt}$)
V_{12}^{zt} (μM/s)	0.015	0.64 ($= V_{21}^{zt}$)
V_{12}^{zt}/K_{12}^{zt} (s^{-1})	$9 \cdot 10^{-3}$	0.11
K^{ee} (μM)	501	88
K_{21}^{zt} (μM)	16.4	–
R_{00} (s/μM)	71.4	3.01
R_{00}/R^{ee}	325	31

extreme directional asymmetry in its operation and the great differential mobility of loaded and empty carrier. In fact, as indicated by the R_{00}/R^{ee} values (Table III), at 5 °C the loaded carrier moved about 325-times more rapidly than the empty carrier (R_{00}/R^{ee}). We have previously [12] demonstrated for erythrocytes from freshly drawn blood that the van't Hoff and Arrhenius plots of K^{ee} and V^{ee}, respectively, are linear between 35 and 5 °C (corresponding to an apparent enthalpy of 5.7 kcal/mole and an activation energy of about 18

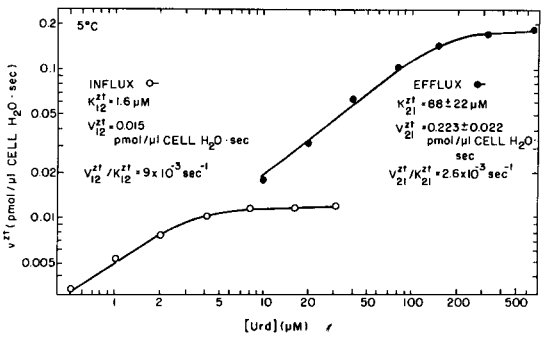

Fig. 5. Comparison of initial velocities of zero-*trans* entry and exit of uridine in outdated red cells at 5 °C. The details of the experiment are described in the legend to Fig. 4.

kcal/mole), but that the plots of $K_{12}^{zt} = K_{21}^{zt}$ and $V_{12}^{zt} = V_{21}^{zt}$, though parallel to those for K^{ee} and V^{ee} between 35 and 15 °C, exhibit a marked downward break between 15 and 5 °C. In terms of the simple carrier model the results imply a steeper (about 2-fold) temperature coefficient for the mobility of the empty carrier than for that of the loaded carrier between 15 and 5 °C. A similar temperature effect seems to apply to outdated red cells (Table III), since estimates from earlier data (Table I) suggested that at 22–25 °C the differential mobility for loaded and empty carrier (R_{00}/R^{ee}) in outdated red cells is only 12–16.

All kinetic differences between fresh and outdated cells are greatly amplified at 5 °C. First, whereas in fresh cells K^{ee} is lower at 5 °C than at 25 °C, K^{ee} for outdated red cells is at least as high at 5 °C as at 25 °C (Table II). On the other hand, V^{ee} for outdated cells is reduced about 50%, so that the first-order rate constant for equilibrium exchange is only 10% of that in fresh red cells. The lower V^{ee} of outdated cells probably reflects, in part at least, a loss of functional transporters, because the number of high-affinity binding sites for NBTI is lower in outdated than fresh cells. We have measured equilibrium binding in six individual batches of both fresh erythrocytes and outdated cells; the mean values ± S.E. were $K_d = 0.43 \pm 0.04$ and 0.74 ± 0.08 nM, respectively, and the number of binding sites = 15 000 ± 800 and 12 400 ± 1200 per cell, respectively. Jarvis et al. [4] did not detect any difference in number of binding sites between fresh and outdated cells (approx. 11 000/cell), but also observed a higher K_d for outdated than fresh red cells (0.97 and 0.31 nM, respectively).

Second, at 5 °C K_{21}^{zt} is much higher for outdated than fresh cells, whereas the opposite is true for K_{12}^{zt} (cf. Table III, Fig. 5). But more importantly, the maximum zero-*trans* flux is much lower in outdated than fresh cells; V_{21}^{zt} is about 65% lower, whereas V_{12}^{zt} is about 97% lower. Overall, the mobilities of loaded and empty carrier are about 50% and 95%, respectively, lower in outdated than fresh cells. Third, at 5 °C maximum efflux in outdated cells exceeds maximum influx about 14-fold (Fig. 5).

We have no explanation for the marked specific decrease in empty carrier mobility induced by a decrease in temperature to 5 °C. Neither can we explain on a molecular level the change in carrier operation associated with storage of the red cells. However, both effects might be related to structural alteration in the plasma membrane induced by temperature or storage, which would suggest that the operation of the carrier is subject to modification by the membrane environment. The changes induced by low temperature and storage, however, are clearly distinct. The low temperature causes a preferential impairment of the movement of the empty carrier in both directions, whereas storage induces an additional directional asymmetry in the mobility of the empty carrier.

Because ATP seems to play a major role in the maintenance of red-cell membrane structure and function [7,8,13], we have determined whether a decrease in intracellular ATP affects the kinetics of uridine transport in these cells, but with negative results. Incubation of fresh red cells in Tris-saline containing 10 mM of each KCN, KF, and iodoacetate at 37 °C for 4 h, which largely depleted the cells of ATP (> 97%, data not shown), reduced uridine transport about 50%, but equilibrium exchange flux and zero-*trans* influx were reduced to about the same extent (Table IV). Efflux was also reduced to a similar extent (data not shown). Thus the treatment seemed to cause a loss of transporters per se, rather than an alteration of its kinetic properties as observed during storage. We conclude that the altered directional mobility of the empty nucleoside transporter in outdated red cells is not caused by a lowering of intracellular ATP. The decreased total uridine transport capacity induced by incubation with KCN, KF and iodoacetate, on the other hand, could reflect a direct modulation of total uridine transport by ATP, as recently proposed for sugar transport in human red cells (Ref. 13; see below), but in our opinion it is more likely caused by an interaction of the iodoacetate with essential sulfhydryl groups on the carrier.

It has recently been reported that equilibrium exchange of 500 mM D-galactose [14] and the infinite-*cis* transport of D-glucose [13] are about 25% and 40%, respectively, lower in outdated than fresh human erythrocytes. A similar decrease in infinite-*cis* transport of D-glucose was induced by incubation of fresh cells with iodoacetamide plus

TABLE IV

EFFECT OF ATP DEPLETION ON URIDINE TRANSPORT IN FRESH HUMAN ERYTHROCYTES

Washed fresh red cells were suspended to $5 \cdot 10^8$ cells/ml in Tris-saline (control) or Tris-saline containing 10 mM KCN, 10 mM KF and 10 mM iodoacetate (treated). The suspensions were incubated at 37 °C and at zero-time and after 4 h of incubation samples thereof were equilibrated to 25 °C and the inward equilibrium exchange and zero-*trans* influx of 2 mM Urd were measured by rapid kinetic techniques. The equilibrium exchange data were analyzed as explained in the legend to Table II with K^{ee} fixed at 700 μM and v^{ee} was calculated as $SV^{ee}/(K^{ee} + S)$. Equation 1 was fitted to the zero-*trans* entry time courses with R^{ee} $(1/V^{ee})$ fixed at the value determined experimentally for the particular cell population and K at 100 μM. v_{12}^{zt} was calculated as $S_1V_{12}^{zt}/(K + S_1)$.

Cells	37 °C time (h)	pmol/μl cell H_2O per s		v^{ee}/v^{zt}
		v^{ee}	v_{12}^{zt}	
Control	0	50.6 ± 5.6	10.0 ± 0.6	5.1
	4	54.5 ± 3.7	10.3 ± 0.6	5.3
		50.5 ± 3.5	11.5 ± 0.7	4.4
Treated	0	43.0 ± 4.4	7.6 ± 0.4	5.6
	4	24.4 ± 0.9	4.0 ± 0.2	6.1
		23.5 ± 0.5	3.5 ± 0.2	6.6

inosine [13]. Reduced D-glucose transport was apparent in isolated ghosts and reversed by sealing the ghosts in the presence of ATP, and the authors, therefore, concluded that glucose transport is modulated by ATP. In a preliminary experiment we examined whether the kinetics of sugar transport in red cells are affected by blood storage in a similar manner as observed for uridine transport. This was not the case. Both equilibrium exchange and zero-*trans* influx of 50 mM 3-*O*-methyl-D-glucose at 25 °C were slightly lower in outdated than fresh erythrocytes (up to 30% in repeated experiments), but both were reduced to a similar extent (data not shown). These results suggest that only the number of sugar transporters may be reduced in outdated cells, but a more detailed comparative kinetic analysis of sugar transport in fresh and outdated cells is required to confirm this conclusion. Nevertheless, the total transport capacities for 3-*O*-methyl-D-glucose and other sugars [13,14] and for uridine (Table II) were reduced to a similar extent in outdated red cells, perhaps suggesting a common cause.

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