Nucleoside transport in human erythrocytes

A simple carrier with directional symmetry in fresh cells, but with directional asymmetry in cells from outdated blood

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Kinetic characteristics of the transport of uridine, a non-metabolized permeant in human erythrocytes, have been compared in erythrocytes from fresh and outdated stored blood. Uridine transport kinetics in fresh cells conformed to the predictions of a simple carrier model operating with directional symmetry, but in erythrocytes from outdated blood the kinetic characteristics of uridine transport were those of an asymmetric system. The latter result agrees with earlier reports by others. The mobility of the loaded and empty carriers differed by about 6- and 12-fold in fresh and outdated blood, respectively.

Although human erythrocytes have for many years been used in investigations of membrane transport mechanisms (for examples, see Ellory & Lew, 1977), few detailed kinetic studies of nucleoside transport in these cells have been reported since the excellent study by Cabantchik & Ginsburg (1977). Prior to 1977, it was well established that human erythrocytes possess a nucleoside-specific membrane transport system which accepts both purine and pyrimidine nucleosides (for references see Young, 1978). Cabantchik & Ginsburg (1977) further demonstrated that a simple carrier mechanism with the property of directional asymmetry could account for the kinetic characteristics of uridine transport in erythrocytes from outdated human blood.

While studying the nucleoside transport properties of human erythrocytes, it became apparent to us that major differences between erythrocytes from fresh and outdated blood existed in the kinetic parameters for the influx, efflux and equilibrium exchange of uridine. Aims of the present investigation were to characterize these differences by utilizing rapid kinetic techniques and to evaluate in fresh and stored cells the binding of a potent, specific nucleoside transport inhibitor, NBMPR. Evidence

demonstrating that NBMPR binding to erythrocyte membranes represents a specific interaction with functional nucleoside transport elements has been reviewed recently (Paterson *et al.*, 1981; Jarvis & Young, 1982). We demonstrate that (i) influx and efflux of the non-metabolized permeant, uridine, were equivalent in fresh erythrocytes, but differed in cells from outdated stored blood, and (ii) NBMPR binding site concentrations were similar in stored and fresh cells. A preliminary abstract of the present study has been published (Jarvis *et al.*, 1982b).

Materials and methods

Erythrocytes

Whole blood samples were collected into heparinized tubes. Outdated human blood (stored at 4°C as packed red cells in citrate/phosphate/dextrose solution of 22–35 days) was obtained from the Blood Transfusion Service of the Canadian Red Cross Society. Erythrocytes were washed three times with phosphate-buffered saline (Dulbecco & Vogt, 1954) and the plasma and buffy coats were discarded. Red cell counts and haematocrit estimations were performed by established methods (Archer, 1965).

Nucleoside transport

All assays were performed at 22°C. Intervals of nucleoside uptake were initiated by mixing equal volumes of cell suspension (20% haematocrit) and incubation medium and terminated by an 'inhibitor-oil stop' method (Pickard & Paterson, 1972; Jarvis

Abbreviations used: NBMPR (nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine; NBTGR (nitrobenzylthioguanosine), 2-amino-6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine.

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et al., 1980, 1982a,b). Previous experiments have demonstrated that incubation times of 3–5 s yield initial rates of nucleoside transport in fresh and outdated stored cells (Cabantchik & Ginsburg, 1977; Jarvis *et al.*, 1980, 1982a; Hammond *et al.*, 1983). In this method, portions of assay mixture were transferred to microcentrifuge tubes containing ice-cold stopping medium (5 μ M-NBTGR) layered on ice-cold *n*-dibutylphthalate. The tubes were immediately centrifuged at 12000 *g* for 10 s. The aqueous and *n*-dibutylphthalate layers were aspirated and the insides of the tubes were wiped dry, leaving a cell pellet at the bottom of the tube. Radioactivity associated with the cell pellet was determined (Young, 1978). Transport rates were calculated after subtraction of ^3H radioactivity which became cell-associated due to non-mediated permeation and entry into the extracellular space of the pellet; these corrections were obtained in each experimental protocol by performing a parallel experiment in the presence of 5 μ M-NBTGR at 0°C. The water space and the extracellular water space in the cell pellets were determined by using $^3\text{H}_2\text{O}$ and [U- ^{14}C]sucrose, respectively, in place of uridine in the uptake assay.

Equilibrium exchange influx

Erythrocytes were 'loaded' with uridine (0.125–10 mM) by incubation with uridine-containing phosphate-buffered saline for 2 h, by which time equilibrium between intracellular and extracellular uridine had occurred. In assays of equilibrium exchange diffusion, to initiate the process, 0.2 ml portions of 'loaded' cell suspension were mixed with phosphate-buffered saline containing the same concentration of [5- ^3H]uridine.

Zero-trans influx

Rates of zero-trans influx of uridine were determined by incubating erythrocytes in [5- ^3H]uridine (0.1–3.0 mM).

Zero-trans efflux

Erythrocytes were equilibrated with [5- ^3H]uridine (0.1–3.0 mM). Extracellular radioactivity was removed by pelleting the cells and removing the supernatant. Immediately after removal of medium by aspiration, 0.4 ml portions of 'empty' medium were added to cell pellets with continuous vortex mixing to initiate intervals of zero-trans efflux. Final haematocrits were 2.5%. Efflux of [5- ^3H]uridine was assayed by following either the appearance of ^3H radioactivity in the medium or the disappearance of ^3H radioactivity from the cell pellet following termination of efflux by the 'inhibitor-oil stop' method. Previous experiments have demonstrated that no significant metabolism of uridine occurs in erythrocytes (Oliver & Paterson, 1971; Jarvis *et al.*,

1982a). The present efflux experiments approximated a zero-trans protocol, in that, at zero-time, the extracellular concentration of [^3H]uridine was approx. 0.5% of the intracellular concentration.

Data evaluation

Kinetic parameters for transport were extracted from rate (*v*) versus permeant concentration (*s*) data by means of the Michaelis–Menten equation, fitting least squares lines to plots of *s/v* versus *s* and of *v/s* versus *v*. Concordance was generally observed between estimates of the kinetic parameters (K_m and V_{max}) from the two methods and it was therefore concluded that variances were essentially constant throughout the experimental concentration range (see e.g. Cabantchik & Ginsburg, 1977).

Binding of NBMPR

Binding of NBMPR to erythrocytes was determined at 22°C as previously described (Hammond *et al.*, 1981).

Chemicals

[G- ^3H]NBMPR (sp. radioactivity 16 Ci/mmol) and [5- ^3H]uridine (sp. radioactivity 20 Ci/mmol) were purchased from Moravsek Biochemicals, Brea, CA, U.S.A. After storage, NBMPR was repurified just before use by h.p.l.c. on a C_{18} μ -Bondapak column (Waters Associates, Milford, MA, U.S.A.) eluted with methanol/water solutions.

Results

In order to determine whether the nucleoside carrier of erythrocytes from fresh blood exhibited directional asymmetry, as Cabantchik & Ginsburg (1977) observed with erythrocytes from outdated blood, we compared the kinetic parameters for zero-trans influx and efflux in fresh and stored cells (Figs. 1 and 2 and Table 1). Zero-trans influx of uridine was saturable and conformed to simple Michaelis–Menten kinetics, with apparent K_m values of 0.15 and 0.12 mM for erythrocytes from fresh and outdated blood (Fig. 1). In contrast, the V_{max} estimate for uridine influx was 3-fold greater in fresh cells than stored cells (111 and 34 mmol/litre of water per h, respectively). In Fig. 2, the concentration-dependence of uridine efflux is compared in fresh and stored cells in plots of *s/v* versus *s*. The parallel plots in Fig. 2 indicate that the maximum velocities for zero-trans efflux were similar in erythrocytes from fresh and stored blood (89 and 100 mmol/litre of water per h, respectively), and that these values were similar to those for zero-trans influx in fresh cells (Table 1). However, apparent K_m values for zero-trans efflux differed in the two types of cells: 0.14 and 0.38 mM for fresh and outdated erythrocytes, respectively (Table 1).

Fig. 1. Concentration dependence of zero-trans uridine influx by human erythrocytes from fresh (●) and stored (○) blood. The curves (panel a) describing the influx of uridine were calculated from the equation:

$$v \text{ (mmol/litre of water per h)} = V_{\text{max.}} \cdot s / (K_m + s)$$

where *v* is the velocity of influx at an extracellular concentration of *s* mM. The kinetic constants so employed (apparent *K_m* 0.15 and 0.12 mM, and *V_{max.}* of 111 and 34 mmol/litre of water per h, for fresh and outdated stored erythrocytes, respectively) were calculated from the least squares analyses of the *s/v* versus *s* plots of the data shown in panel (b).

Table 1. Comparison of kinetic data for uridine equilibrium exchange, zero-trans influx and zero-trans efflux in human erythrocytes from fresh and outdated stored blood

Data from experiments illustrated in Figs. 1–4 were included in the summary data here presented. Values are means ± S.E.M. with the number of observations in parentheses.

Conditions	Parameter measured	Value for:	
		Fresh blood	Outdated blood
Zero-trans influx	<i>V_{max.}</i> (mmol/litre of water per h)	116 ± 14 (10)	35.4 ± 3.9 (4)
	<i>K_m</i> (mM)	0.17 ± 0.02 (3)	0.13 ± 0.03 (4)
	<i>V_{max.}</i> / <i>K_m</i> (h ⁻¹)	680 ± 70 (3)	330 ± 61 (4)
Zero-trans efflux	<i>V_{max.}</i> (mmol/litre of water per h)	135 ± 16 (4)	117 ± 18 (4)
	<i>K_m</i> (mM)	0.14 ± 0.02 (4)	0.38 ± 0.10 (4)
	<i>V_{max.}</i> / <i>K_m</i> (h ⁻¹)	1006 ± 360 (4)	375 ± 100 (4)
Equilibrium exchange influx	<i>V_{max.}</i> (mmol/litre of water per h)	462 ± 60 (6)	355 ± 41 (4)
	<i>K_m</i> (mM)	0.76 ± 0.07 (6)	1.10 ± 0.10 (4)
	<i>V_{max.}</i> / <i>K_m</i> (h ⁻¹)	640 ± 105 (6)	350 ± 70 (4)

In equilibrium exchange experiments, substrate concentrations were identical on both sides of the membrane. In this condition, inward fluxes of uridine in erythrocytes from fresh and outdated blood were, respectively, approx. 4 and 10 times faster than inward zero-trans fluxes. Observed were apparent *K_m* values [means ± S.E.M. (*n*)] of 0.76 ± 0.07 (6) and 1.1 ± 0.1 (4) mM for isotope exchange in the inward direction in cells from fresh and outdated blood, respectively, and *V_{max.}* estimates of 462 ± 60 (6) and 355 ± 41 (4) mmol/litre of water per h (Table 1).

Data from a representative experiment are shown in Fig. 3.

In order to test whether the observed decrease in *V_{max.}* for zero-trans uridine influx in stored cells was due to a decreased number of functional nucleoside transporters, the high-affinity, site-specific binding of NBMPR (Jarvis & Young, 1980; Cass *et al.*, 1981) to fresh and stored erythrocytes was compared. Observed densities of NBMPR binding sites on erythrocytes from fresh and outdated blood were similar [11000 ± 600 (9) and 12000 ± 1000 (6)

Fig. 2. Concentration dependence of zero-trans uridine efflux from human erythrocytes from fresh and stored blood

Data are as s/v versus s plots. Linear least squares analyses of these data yielded the following kinetic constants: apparent K_m values 0.12 and 0.34 mM, and V_{max} values of 89 and 100 mmol/litre of water per h for fresh (panel a) and outdated stored (panel b) erythrocytes, respectively.

sites/cell, respectively]. However, the apparent affinity for NBMPR of the binding sites on erythrocytes from fresh blood was higher than that of the sites on stored cells. Observed were apparent K_D values (nM) of 0.31 ± 0.02 (9) and 0.97 ± 0.13 (6) (the experiment of Fig. 4 is included in these data).

Discussion

The present results demonstrate that (i) the kinetic properties of the uridine transport mechanism of fresh erythrocytes are consistent with those expected of a simple symmetric carrier (Lieb & Stein, 1974), and (ii) this mechanism in erythrocytes from outdated stored blood exhibits directional asymmetry.

Fig. 3. Effect of extracellular concentration of uridine on uridine influx in equilibrium exchange in human erythrocytes from fresh (●) and stored (○) blood

Linear least squares analyses of the plots yielded apparent K_m values of 0.63 and 1.33 mM, and V_{max} values of 350 and 380 mmol/litre of water per h, for fresh and outdated stored erythrocytes, respectively.

Fig. 4. Mass law analysis of site-specific binding of NBMPR to human erythrocytes from fresh (●) and stored (○) human blood

Human erythrocytes were incubated for 20 min with medium containing various concentrations of [$G-^3H$]NBMPR in the presence or absence of $5 \mu M$ -NBTGR. Cells were pelleted and supernatant fluids assayed to determine the 'free' NBMPR concentration. After washing the cells once with 1 ml of ice-cold medium, radioactivity associated with the cell pellet was determined. 'Bound' NBMPR refers to site-specific binding, that is, to differences in cell content of NBMPR in the presence and absence of $5 \mu M$ -NBTGR.

For a facilitated transport system to be compatible with the simple carrier model, the $V_{\max.}/K_m$ ratios for all experimentally measured fluxes must be equal (Lieb & Stein, 1974). The data of Table 1 demonstrate that, within experimental error, the uridine transport system of erythrocytes from fresh and outdated blood displays this property. $V_{\max.}/K_m$ ratios were approximately 775 and 352h^{-1} , respectively, for cells from fresh and outdated blood. The kinetic constants for zero-*trans* entry and exit (see Table 1) were equivalent for fresh erythrocytes, indicating directional symmetry in the transport of uridine. In contrast, uridine transport in erythrocytes from outdated blood exhibited marked directional asymmetry in that zero-*trans* influx and efflux were not comparable (see Table 1). The latter result is consistent with previously published data (Cabantchik & Ginsburg, 1977). Assuming the validity of the simple carrier model, the difference in zero-*trans* entry and exchange data imply that the mobility [carrier mobility implies macromolecular movement, e.g. a conformational shift, and does not necessarily denote translocation of the carrier] of the loaded and empty nucleoside carriers differs by about 6- and 12-fold in fresh and outdated blood, respectively. This finding, together with the observation that cells from fresh and outdated blood have similar numbers of binding sites for NBMPR, suggests that the decrease in the $V_{\max.}$ value for zero-*trans* influx in stored erythrocytes, relative to that in fresh cells, may be attributed to a decreased mobility of the unloaded carrier.

The differential mobilities of loaded and empty nucleoside carriers observed in erythrocytes is not seen in cultured mammalian cells (Wohlhueter *et al.*, 1979). This fact and the results here reported suggest that cellular nucleoside transport activity might possibly be regulated by varying the mobilities of the loaded and unloaded carriers. The erythrocyte nucleoside transport system would appear to be a system in which this concept may be tested and which affords an opportunity to determine the factors responsible for variations in carrier mobility.

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