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Nucleoside transporter of pig erythrocytes. Kinetic properties, isolation and reaction with nitrobenzylthioinosine and dipyridamole

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Rapid kinetic techniques were used to measure the transport of uridine in pig erythrocytes in zero-*trans* entry and exit and equilibrium exchange protocols. The kinetic parameters were computed by fitting appropriate integrated rate equations to the time-courses of transmembrane equilibration of radiolabeled uridine. Transport of uridine conformed to the simple carrier model with directional symmetry, but differential mobility of substrate-loaded and empty carrier. At 5 °C, the carrier moved about 30-times faster when loaded than when empty. Uridine transport was inhibited in a concentration-dependent manner by nitrobenzylthioinosine and dipyridamole and the inhibition correlated with the binding of the inhibitors to high-affinity binding sites on the cells (K_d about 1 and 10 nM, respectively). Thus, in its kinetic properties, differential mobility when empty and loaded, and sensitivity to inhibition by nitrobenzylthioinosine and dipyridamole, the transporter of pig erythrocytes is very similar to that of human erythrocytes. Also, the total number of high-affinity binding sites for nitrobenzylthioinosine and dipyridamole/cell were similar for the two cell types and the [^{3}H]nitrobenzylthioinosine-labeled carrier of pig erythrocytes, just as that of human red cells, was mainly recovered in the band 4.5 protein fraction of Triton X-100-solubilized membranes. However, sodium dodecylsulfate-polyacrylamide gel electrophoresis of photoaffinity-labeled band 4.5 membrane proteins indicated a slightly higher molecular weight for the transporter from pig than human erythrocytes. We have also confirmed the lack of functional sugar transport in erythrocytes from adult pigs by measuring the uptake of various radiolabeled sugars. But in spite of the lack of functional sugar transport we recovered as much band 4.5 protein from pig as from human erythrocyte membranes.

Introduction

Mammalian cells possess a facilitated nucleoside transport system with broad substrate specificity [1,2]. It transports all natural ribo- and deoxyribonucleosides and many analogs thereof, albeit with different efficiencies [1–3]. Two forms of nucleoside transport, however, can be distinguished on the basis of sensitivity to inhibition by nitrobenzylthioinosine (NBTI) [4–6]. One form is strongly inhibited by nanamolar concentrations of NBTI (designated NBTI-sensitive), resulting from the binding of NBTI to high-affinity binding sites on the plasma membrane ($K_d \approx 1$ nM). The other form is not associated with such binding sites and is inhibited only by micromolar concentrations of NBTI (designated NBTI-resistance). Human erythrocytes and some cultured cell lines

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express only NBTI-sensitive nucleoside transport, whereas some cell lines express only NBTI-resistant transport and the majority of cell lines investigated express both types in various proportions [4–8].

Kinetically, both forms behave as simple symmetrical carriers and exhibit similar substrate specificity and kinetic properties [1,9,10]. Thus, it is possible that they represent the same gene product that exhibits either NBTI-resistance or sensitivity due to differences in membrane organization or association with other lipophilic membrane components, which are requisite to formation of the high affinity NBTI binding sites [2,11]. In this case, an alteration in membrane structure of the lipophilic components of the high-affinity NBTI binding site could account for the apparent genetic basis of NBTI-resistant and -sensitive nucleoside transport [12–14].

By photoaffinity labeling with [^{3}H]NBTI and reconstitution experiments, the nucleoside transporter of human erythrocytes has been identified as 45–60 kDa glycoprotein of the band 4.5 membrane protein fraction [15,16], and similar proteins have been photoaffinity-labeled with [^{3}H]NBTI in other mammalian cells [17–20]. Isolation of the nucleoside transporter from the human red cell membrane is complicated by the fact that the sugar transporter is also a band 4.5 protein [21,22] and, based on the number of high-affinity NBTI and cytochalasin B binding sites, is present at 10–20-times higher concentrations than the nucleoside transporter [23–26]. A more appropriate source for purifying the nucleoside transporter might be erythrocytes from adult pigs, since these cells exhibit a similar level of nucleoside transport activity as human erythrocytes [27,28], but seem to lack a functional sugar transporter [29]. However, the [^{3}H]NBTI-photoaffinity labeled band 4.5 proteins of pig erythrocytes have been reported to possess a slightly higher molecular weight than the analogous proteins from human erythrocytes [15]. They have also been reported [30,31] to adsorb, when detergent-solubilized, to DEAE-cellulose, whereas the human nucleoside transporter is not adsorbed [15,16], and a recent preliminary report [32] has indicated that monoclonal antibodies to the band 4.5 proteins of pig erythrocytes precipitate [^{3}H]NBTI-photoaffinity labeled pig band 4.5 proteins, but not the analogous photoaffinity-labeled proteins from human erythrocyte membranes. These results suggest that the nucleoside transporters from the two types of erythrocytes differ structurally and antigenically and possibly in other properties. We have, therefore, undertaken a detailed comparison of the kinetic and molecular properties of the nucleoside transporters from pig and human erythrocytes and their interaction with NBTI and dipyridamole, another potent inhibitor of nucleoside transport in human erythrocytes and other mammalian cells [1,2].

Experimental Procedures

Pig erythrocytes. Fresh blood from adult pigs was kindly suppled by Dr. T. Molitor (School of Veterinary Medicine, University of Minnesota). The erythrocytes were thrice washed in cold phosphate-buffered saline with removal of the lymphocyte containing buffy coat and suspended in saline containing 5 mM Tris-HCl (pH 7.4; Tris-saline). The cells were enumerated with a Coulter counter.

Transport measurements. Except where indicated otherwise, time-courses of transmembrane equilibration of radiolabeled uridine in erythrocytes were measured under zero-*trans* and equilibrium exchange conditions by rapid kinetic techniques using a dual syringe apparatus (15 time points/ time-course) as described previously [1,10,33]. At specified short time intervals of incubation at 25 °C, the cells were separated from the medium by centrifugation through an oil layer and analyzed for radioactivity. Radioactivity in cell pellets was corrected for that trapped in extracellular space as estimated with [^{14}C]inulin [34] and converted to pmol/μl cell water on the basis of the intracellular water space determined in each experiment by use of ^3H_2O [34].

For the kinetic analysis of uridine transport in pig erythrocytes, which exhibits directional symmetry but differential mobility of loaded and empty carrier (see later), inward equilibrium exchange and zero-*trans* influx were measured each at seven uridine concentrations in the same population of cells [10]. Samples of suspensions of $6 \cdot 10^8$ to $1 \cdot 10^9$ cells/ml were mixed with solu-

tions of radiolabeled uridine with the dual syringe apparatus (5-ml and 1-ml syringes) in a ratio of 7.3 : 1. For equilibrium exchange measurements, the cells were preloaded to equilibrium (about 1 h at 37 °C) with specified concentrations of unlabeled uridine. The following equation was fitted to the pooled equilibrium exchange data

$$N_{2,t} = N_2\left[1-\exp\left(-\frac{V^{ee}t}{K^{ee}+S}\right)\right] \quad (1)$$

where $N_{2,t}$ is the intracellular concentration of radiolabel at time t; N_2 is the equilibrium concentration of radioactivity in the cells, which was equal to the radioactivity in an equivalent volume of medium; S is the chemical concentration of substrate; and K^{ee} and V^{ee} are the apparent Michaelis-Menten constant and maximum velocity for equilibrium exchange, respectively. The analysis yields best fitting parameters of K^{ee} and V^{ee}. Then the following equation was fitted to the pooled zero-*trans* data with R_{ee} $(=1/V^{ee})$ fixed at the value experimentally determined for the cell population being analyzed and with R_{12} held equal to R_{21} (directional symmetry)

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

where $S_{2,t}$ is the concentration of intracellular substrate at time t $(S_{2,0}=0)$; S_1 is the extracellular concentration of substrate (taken as constant); and the R terms are resistant factors, proportional to the round trip time of the carrier in four modes [1,9]; empty in both directions (R_{oo}), loaded in both directions (R_{ee}), empty inward and loaded outward (R_{21}) and loaded inward and empty outward (R_{12}). The latter analysis yields best-fitting parameters of K, the limit Michaelis-Menten constant, and $R_{12}=R_{21}$ $(=1/V^{zt}$, the maximum velocity of zero-*trans* entry and exit) and permits calculation of R_{oo} $(=R_{12}+R_{21}-R_{ee})$ and of the zero-*trans* and equilibrium exchange Michaelis-Menten constants $K_{12}^{zt}=KR_{oo}/R_{12}$ and $K^{ee}=KR_{oo}/R_{ee}$, respectively [1,9]. The ratio of R_{oo}/R_{ee} quantitates the differential mobility of loaded and empty carriers [10,33]. Only when loaded and empty carrier mobilities are equal does $R_{ee}=R_{oo}$. On the other hand, $R_{12}=R_{21}$ indicates directional symmetry of the carrier [9,10]. Initial zero-*trans* entry (v_{12}^{zt}) and equilibrium exchange velocities (v^{ee}) were calculated for a given substrate concentration as the slopes of the curves described by Eqns. 2 and 1, respectively, for $t=0$ [1].

In all experiments where only v^{ee} at a single permanent concentration was of interest, Eqn. 1 was fitted to the data with K^{ee} fixed at 700 μM (25 °C; see later), except where indicated otherwise. For measuring the effects of NBTI and dipyridamole on uridine exchange, these were added to cell suspensions (at 25 °C) at least 2 min prior to measuring the exchange of radiolabeled uridine. For measuring the effects of sugars on uridine exchange, these were added simultaneously with radiolabeled uridine.

For exit measurements, an undiluted suspension of erythrocytes (about $5\cdot10^9$ cells/ml) was equilibrated with 2.5 mM [^{3}H]uridine. Samples of the suspension of preloaded cells were mixed with the dual syringe apparatus in short time intervals in a ratio of 1 : 7 : 3 (opposite to that in entry measurements) with Tris-saline devoid of uridine (zero-*trans* exit) or Tris-saline containing 2.5 mM unlabeled uridine (outward equilibrium exchange).

Equilibrium binding of NBTI and dipyridamole. Equilibrium binding of ^{3}H-labeled ligands was measured as described previously [11,35]. The following equation was fitted to concentrations of bound ligand (L_b; measured as total ligand minus free ligand) and free ligand (L_f)

$$L_b = \frac{NL_f}{K_d+L_f}+k'L_f \quad (3)$$

where N is the number of binding sites per liter; K_d is the dissociation constant and k' is a coefficient of non-saturable binding.

Data analysis. The theoretical equations were fitted to data by a generalized least-squares regression program based on the algorithm of Dietrich and Rothmann [36]. Parameter values are reported ± S.E. of the least-square estimate unless indicated otherwise.

Isolation of band 4.5 proteins. Band 4.5 proteins of human erythrocyte membranes were isolated as described by Kasahara and Hinkle [21]. In brief, white ghosts were isolated from erythrocytes of

outdated human blood and stripped of peripheral proteins by treatment with 0.1 mM EDTA (pH 11). The stripped membranes were solubilized in 1% Triton X-100 and passed through a DEAE-cellulose column. Over 90% of the protein in the flow-through fraction represented band 4.5 proteins and essentially all of the material with high affinity NBTI binding sites was recovered in this fraction (Woffendin et al., submitted for publication). The band 4.5 proteins were isolated from pig erythrocyte membranes as described for human band 4.5 proteins.

Photoaffinity-labeling with [^{3}H]NBTI. Stripped membranes suspended in 50 mM Tris-HCl (pH 7.4) containing 10 mM dithiothreitol at a concentration of about 5 mg protein/ml were incubated with 10 nM [^{3}H]NBTI at room temperature for 30 min and then irradiated for 2 min at a distance of 4 cm with a 8W short-wavelength ultraviolet light (MR4, G.W. Gates and Co., Inc., Long Island, NY). The membranes were washed thrice with 100 volumes of 50 mM Tris-HCl (pH 7.4) containing 5 μM unlabeled NBTI.

Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE). Stripped membranes and band 4.5 proteins were analyzed by SDS-PAGE using 10% gels as described by Laemmli [37].

Materials. [5-^{3}H]Uridine, [G-^{3}H]NBTI and [*piperidyl*-^{3}H]dipyridamole were purchased from Moravek Biochemicals (Brea, CA) and diluted to the desired specific radioactivities with unlabeled uridine, NBTI or dipyridamole. Unlabeled nucleosides and sugars were obtained from Sigma (St. Louis, MO) and unlabeled NBTI from Calbiochem (San Diego, CA) and SM-2 Bio-Beads and DEAE-cellulose (Cellex D) from Bio-Rad (Richmond, CA). Dipyridamole (Persantin) was a gift from Geigy Pharmaceuticals (Yonkers, NY).

Results and Discussion

Kinetic properties of pig red cell nucleoside transport system

Our first concern was whether the directional symmetry of uridine transport observed in human erythrocytes and mammalian cell lines applied to pig erythrocytes. The results in Fig. 1 illustrate that this was the case. At a concentration of uridine (3 mM) well above the Michaelis-Menten

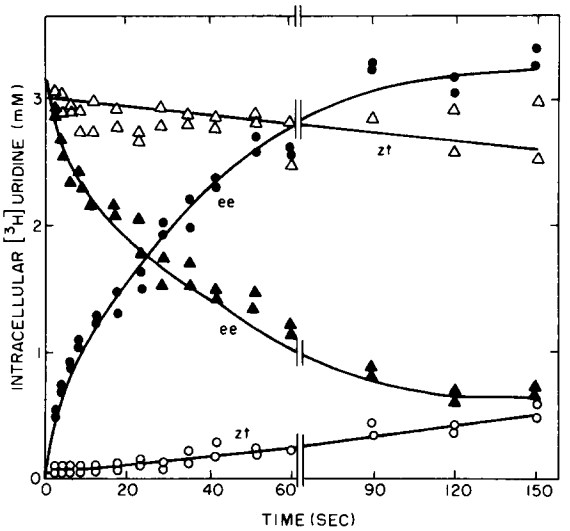

Fig. 1. Zero-*trans* exit and entry and inward and outward equilibrium exchange of 2.5 mM uridine in pig erythrocytes at 25° C. Time-courses of zero-*trans* entry (○———○) and exit (△———△) and inward (●———●) and outward (▲———▲) equilibrium exchange were determined in duplicate by rapid kinetic techniques as described under Experimental Procedures.

constants reported for uridine transport in other mammalian cells, the initial velocities of zero-*trans* entry and exit at 25° C were about equal within experimental errors ($v_{12}^{zt} = v_{21}^{zt}$). Furthermore, the data in Fig. 1 show that equilibrium exchange flux was much faster than zero-*trans* flux. This difference is a clear sign that the substrate-loaded carrier moves more rapidly than the empty carrier [9,10] as it is observed for the nucleoside transporter of human erythrocytes [10,24]. This property distinguishes it from nucleoside transporters of cultured mammalian cells, which exhibit equal mobility when loaded and empty [1,38]. The differential mobility of loaded and empty nucleoside carrier of human erythrocytes is particularly striking at lower temperatures, such as 5° C [33]. Thus, for a comparison of the differential mobilities of the nucleoside transporters of human and pig erythrocytes, we have determined the kinetic parameters of uridine transport in pig erythrocytes at 5° C. Inward equilibrium exchange and zero-*trans* entry were measured in the same population of pig erythrocytes at several uridine concentrations and the data analyzed (Table I) as previously applied to human erythrocytes [10,33]. Eqn. 1 was

TABLE I

COMPARISON OF KINETIC PARAMETERS FOR URIDINE TRANSPORT IN PIG AND HUMAN ERYTHROCYTES AT 5 °C

The equilibrium exchange (ee) and zero-*trans* (zt) influx of uridine were measured in a suspension of $1 \cdot 10^9$ pig erythrocytes/ml at seven concentrations ranging from 10 to 640 μM and 0.5 to 32 μM, respectively, by rapid kinetic techniques, and the data were analyzed by integrated rate analysis as described under Experimental Procedures and in the text. The results for human erythrocytes are from Ref. 33.

Experimental protocol	Parameter	Erythrocytes	
		Pig	Human
ee	K^{ee} (μM)	250 ± 19	279 ±23
	V^{ee} (μM/s)	3.93± 0.14	10.4± 0.4
	V^{ee}/K^{ee} (s^{-1})	0.016	0.037
	R_{ee} (s/mM)	254	95.8
zt	$R_{12} = R_{21}$ (s/mM)	4920 ±115	1553 ±42
	K (μM)	4.8 ± 0.3	2.8± 0.3
	$K_{12}^{zt} = K_{21}^{zt}$ (μM)	9.4	5.4
	K^{ee} (μM)	183	88
	$V_{12}^{zt} = V_{21}^{zt}$ (μM/s)	0.203	0.64
	V^{zt}/K^{zt} (s^{-1})	0.023	0.11
	R_{oo} (s/mM)	9580	3010
	R_{oo}/R_{ee}	38	31

fitted to the equilibrium exchange data to estimate the Michaelis-Menten constant and maximum velocity for exchange, K^{ee} and V^{ee} ($= 1/R_{ee}$), respectively. The integrated zero-*trans* equation (Eqn. 2) was fitted to the zero-*trans* data with R_{ee} fixed at the value measured for the same cell population and with R_{12} and R_{21} held equal (as justified by the observed directional symmetry of the carrier). This analysis yielded estimates of K and $R_{12} = R_{21}$ from which the Michaelis-Menten constant and maximum velocity of zero-*trans* entry and exit, $K_{12}^{zt} = K_{21}^{zt}$ and $V_{12}^{zt} = V_{21}^{zt}$, respectively, and R_{oo} were calculated. Representative initial time-courses of transmembrane equilibration of [^{3}H]uridine under equilibrium exchange and zero-*trans* conditions at 5 °C are shown in Fig. 2. The best fitting kinetic parameters are presented in Table I and compared to those obtained in a previous study for uridine transport in human erythrocytes. The kinetic properties of uridine transport in the two types of erythrocytes were strikingly similar. The Michaelis-menten constants for equilibrium exchange (K^{ee}) and the

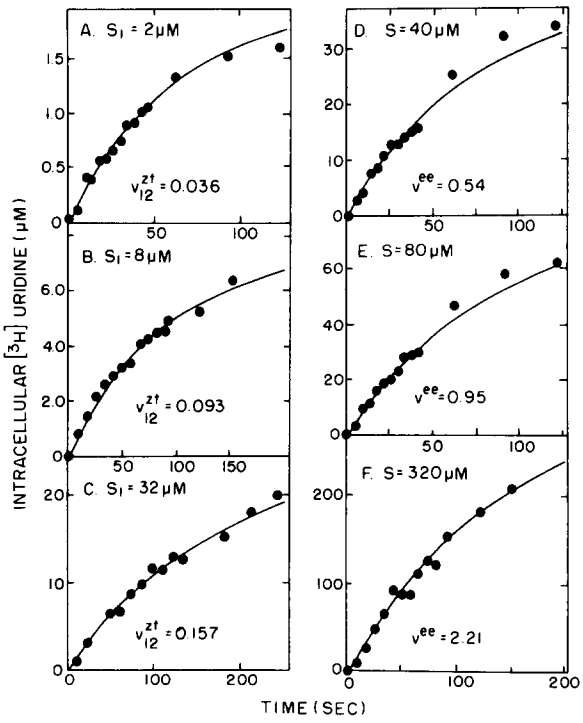

Fig. 2. Zero-*trans* entry (A–C) and inward equilibrium exchange (D–F) of various concentrations of uridine by pig erythrocytes at 5 °C. The details of the experiment are described in the legend to Table I. Representative theoretical curves for zero-*trans* entry are shown in frames A–C ($S_1 = 2$, 8 and 32 μM) and for equilibrium exchange in frames D–F ($S = 40$, 80 and 320 μM). v_{12}^{zt} and v^{ee} were calculated from the fitted parameters for the given substrate concentrations as the slopes of the theoretical curves at $t = 0$ and are expressed in pmol/μl cell water per S.

limit Michaelis-Menten constants (K) were about the same for the two cell types as was the differential mobility of loaded and empty carrier, which was quantified by the R_{oo}/R_{ee} value. At 5 °C, the loaded carriers of both types of cells moved 30–40-times more rapidly than the empty carrier. The maximum velocities were somewhat lower for the pig than the human erythrocytes, but whether this difference is significant is uncertain, since maximum velocities can vary considerably between different batches of human red cells [10]. In part, the difference might also reflect the smaller size (10–20%) of the pig erythrocytes. The validity of the kinetic analyses, based on the simple carrier model, is indicated by the equality within experimental errors of the V^{ee}/K^{ee} and V^{zt}/K^{zt} ratios, which is required by this model, as well as by the

finding that K^{ee} estimated from the zero-*trans* data was similar to K^{ee} estimated directly (Table I).

The similarity of the kinetic properties of the nucleoside transporters of pig and human erythrocytes extended to 25°C. The best fitting parameters for uridine transport in pig erythrocytes at 25°C as determined in one experiment were: $K^{ee} = 760 \pm 72$ μM; $V^{ee} = 88 \pm 3$ pmol/μl cell water per s; $K = 69 \pm 6$ μM; and $R_{12} = R_{21} = 11.4 \pm 3.0$ s/mM. These values are similar to those observed for uridine transport in human erythrocytes at this temperature [10,33]. The K^{zt} and V^{zt} values computed by the integrated rate analysis described above (69 μM and 9.3 pmol/μl cell water per s, respectively) were lower than those reported previously for uridine transport in pig erythrocytes (250 μM and 15 pmol/μl cell water per s, respectively), which were calculated from initial velocities (estimated from single 3-s time points of uridine uptake) without taking the differential mobilities of loaded and empty carrier into consideration [27].

Inhibition of nucleoside transport by NBTI and dipyridamole

The equilibrium exchange of 500 μM uridine by pig erythrocytes was inhibited in a concentration dependent manner by NBTI and dipyridamole (Fig. 3). The concentrations of both inhibitors causing 50% inhibition (IC_{50}) were similar to those observed for human erythrocytes (Ref. 23 and Fig. 3B). However, they were significantly lower than the values reported previously for the inhibition of zero-*trans* entry of 1 mM uridine into pig erythrocytes by NBTI and dipyridamole (70 and 500 nM, respectively; Ref. 28). This discrepancy is explained by procedural differences in the two studies. In the study by Jarvis et al. [28], the inhibitors were added to the cells simultaneously with radiolabeled uridine. However, at the lower concentrations used in these experiments both NBTI and dipyridamole equilibrate only relatively slowly with the high affinity binding sites on both human erythrocytes and cultured cells lines (Refs. 11, 35, 39, and Woffendin and Plagemann (unpublished data)) so that maximum inhibition is attained only after several seconds of incubation at 25°C (Refs. 35, 40, and Woffendin

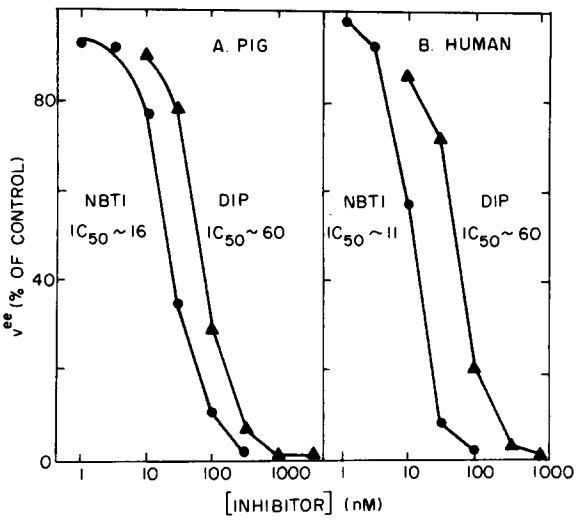

Fig. 3. Effects of NBTI and dipyridamole on uridine equilibrium exchange by pig (A) and human (B) erythrocytes at 25°C. A. Samples of a suspension of $1 \cdot 10^9$ pig erythrocytes/ml that had been equilibrated with 500 μM unlabeled uridine were supplemented with the indicated concentrations of NBTI or dipyridamole. After at least 2 min of incubation, the transmembrane equilibration of 500 μM [^{3}H]uridine (0.24 cpm/pmol) was measured by rapid kinetic techniques as described under Experimental Procedures. Eqn. 1 was fitted to the data with K^{ee} fixed at 700 μM (see text) and the v^{ee} values were calculated as the slopes to the progress curves at $t = 0$. v^{ee} for the control suspension was 49.4 ± 2.3 pmol/μl cell water per s. Experiments with human erythrocytes (B) were conducted in the same manner and represent data similar to those reported previously [46,58].

and Plagemann (unpublished data)). Thus, for determining maximum effects of the inhibitors, we have preincubated the cells with various concentrations of the inhibitors for several minutes before measuring the exchange of uridine. Uridine transport was completely inhibited by 300 nM NBTI (as well as by 1 μM dipyridamole), which indicates that pig red cells express only NBTI-sensitive nucleoside transport, just as do human erythrocytes [7,23]. The nucleoside transporters of pig and human erythrocytes share a relatively high sensitivity to inhibition by dipyridamole, which is significantly higher than that of transporters of several cell lines (IC_{50} = 100–1000 nM; Ref. 5).

The dissociation constants (K_d) for the binding of NBTI (about 1 nM) and of dipyridamole (about 10 nM) to pig red cells and the apparent number of high-affinity binding sites (Fig. 4) were also comparable to the values reported for human red

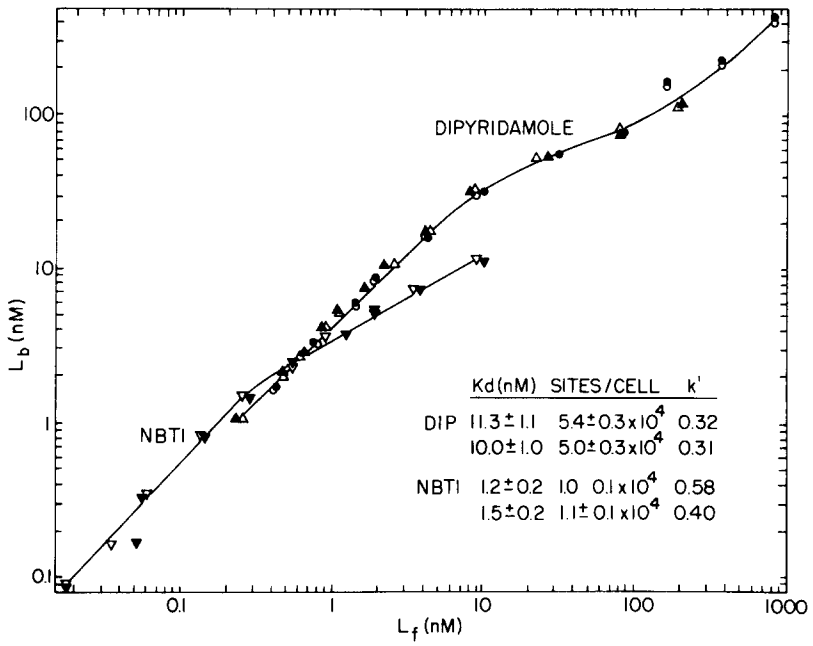

Fig. 4. Amounts of [^{3}H]NBTI (▽———▽, ▼———▼) and [^{3}H]dipyridamole (●———●, △———△, ▲———▲) bound (L_b) at equilibrium by pig erythrocytes as a function of free ligand (L_f). Samples of suspensions of about $8 \cdot 10^8$ cells/ml were mixed with equal volumes of 12 solutions containing [^{3}H]NBTI or [^{3}H]dipyridamole and unlabeled ligands to yield final concentrations of 0.1 to 21 nM and 3 μM NBTI, and 2 to 640 nM and 10 μM dipyridamole. After 20 min of incubation at room temperature, the suspensions were centrifuged in an Eppendorf microfuge at 12000 rpm for 5 s. Duplicate samples of the supernatant fluid as well as of the original solutions of radiolabeled ligands were analyzed for radioactivity (free and total ligand, respectively). Eqn. 3 was fitted to the data and the best fitting parameters for two experiments with each ligand (indicated by different symbols) are listed.

cells [23–25]. The K_d values correlated with the observed inhibition of uridine exchange in both types of cells considering that the v^{ee} values in Fig. 3 are plotted as a function of the concentration of total rather than of free inhibitor. Under the conditions of the experiment ($8 \cdot 10^8$ to $1 \cdot 10^9$ cells/ml), 60 to 90% of the total inhibitor added became bound to cell material so that the IC_{50} values, when expressed on the basis of free inhibitor, were about 2–3 nM and about 12 nM for NBTI and dipyridamole, respectively.

The correlation between the K_d of equilibrium binding and the inhibition of uridine transport for both inhibitors is consistent with the view that the transport inhibition by nanamolar concentrations of both results from their interaction with high-affinity binding sites on the cells, but the molecular nature of the binding sites has not been elucidated. Nevertheless, that the substrate binding site of the nucleoside transporter is a component of both the high-affinity NTBI and dipyridamole binding sites is indicated by several lines of evidence. Binding of NBTI to a number of different mammalian cells is competitively inhibited by various nucleosides and other inhibitors of nucleoside transport, such as dipyridamole and dilazep [6,39,41–43] and so is the photoaffinity labeling of band 4.5 protein of human erythrocytes by [^{3}H]NBTI [15]. In fact, the K_i of inhibition of NBTI binding to human erythrocytes by dipyridamole (4.5 nM; Ref. 41) is about the same as the K_d for [^{3}H]dipyridamole equilibrium binding to human and pig erythrocytes. Similarly, we have observed that the equilibrium binding of [^{3}H]dipyridamole to human erythrocytes is inhibited by nucleosides, NBTI, dilazep and lidoflazine [35]. In fact, 12 mM uridine and 6 μM NBTI completely inhibited the binding of [^{3}H]dipyridamole to high-affinity sites of these cells as well as the photoaffinity labeling of the band 4.5 proteins with [^{3}H]dipyridamole, which indicates that high-affinity dipyridamole binding is exclu-

sively to sites associated with the nucleoside carrier. Uridine and NBTI similarly inhibited the high-affinity binding of [^{3}H]dipyridamole to pig erythrocytes (data not shown). The finding that NBTI and dipyridamole inhibit the binding of each other may suggest that they are binding to the same sites. However, some results suggest that the sites for NBTI and dipyridamole may not be identical, although they clearly overlap. For example, dipyridamole at micromolar concentrations only partially displaces bound [^{3}H]NBTI from human erythrocytes [6] and hamster cells [11] and inhibits the displacement of bound [^{3}H]NBTI by unlabeled NBTI [39,44,45]. Furthermore, both NBTI-resistant and -sensitive nucleoside transport are similarly inhibited by dipyridamole [5].

Also, the apparent number of high affinity binding sites for NBTI ($(1–1.5) \cdot 10^4$/cell) and dipyridamole (about $5 \cdot 10^4$/cell) of both human [35] and pig erythrocytes (Fig. 4) differ significantly. The results raise the possibility that more than one molecule of dipyridamole binds per nucleoside carrier, whereas a ratio of 1 : 1 is assumed for NBTI binding [2,7,15,16,24]. On the other hand, the higher estimate for the number of dipyridamole than NBTI binding sites could simply reflect an overestimation that is caused by the relatively high level of non-specific and especially of low-affinity binding of dipyridamole to the cells (Fig. 4; Ref. 35). The presence of dipyridamole binding sites with lower affinity is clearly indicated by a further apparent saturation of binding in the 1 to 50 μM range (data not shown). These sites may represent other carriers, since dipyridamole inhibits the transport of other substances besides nucleosides, such as sugars and phosphate [1]. The IC_{50} for the inhibition of sugar transport by dipyridamole in human red cells is about 100-times higher than that for the inhibition of uridine transport [46] and thus suggests a rather low-affinity interaction of dipyridamole with the sugar transporter. However, whether interaction with a sugar transporter plays a role in the binding of dipyridamole to erythrocytes from adult pigs is uncertain since these cells lack functional sugar transporters (Ref. 29, and see later).

The number of high-affinity NBTI binding sites were somewhat lower for pig than human erythrocytes. This finding may be related to a smaller size of pig erythrocytes referred to already. Thus, based on the V^{ee} values for uridine exchange at 5 °C and 25 °C and the number of NBTI binding sites/cell, the turnover numbers for the carrier from human and pig erythrocytes are comparable. This conclusion confirms an earlier report [27] based on maximum velocities of zero-*trans* entry of uridine.

Sugar uptake by pig erythrocytes

The conclusion that adult pig erythrocytes lack a functional sugar transporter was based on the finding that these cells, when intact, fail to glycolyze D-glucose, but do so after they have been permeabilized by treatment with amphotericin B [29]. Not explained, however, was the finding that under similar conditions, intact pig red cells glycolyzed D-ribose, though inefficiently [47]. Why the pig cells apparently take up D-ribose, but not D-glucose, is unclear, since results with human erythrocytes indicate that both are transported by the same carrier and, in fact, D-ribose is transported less efficiently than D-glucose [48,49]. We have therefore directly compared the uptake of various radiolabeled sugars by adult pig erythrocytes. Sugars entered the cells only very slowly when compared to uridine transmembrane equilibration; the first order rate constants for D-glucose and 3-*O*-methyl-D-glucose uptake (v/S) were about three orders lower than that for uridine exchange (V^{ee}/K^{ee}). The rates of uptake of various sugars, measured over a 1 h period, are summarized in Table II and are compared to the lipid solubilities of the sugars, which were estimated by their solubility in octanol as compared to that in an aqueous solution. The finding that the rates of entry of D-glucose, D-galactose, D-ribose and D-xylose, which are substrates for the sugar transporters of other mammalian cells, were similar to that of L-glucose,which is not a substrate, indicates their entry was primarily non-mediated. The slight differences in uptake rates of the sugars are explained by differences in their lipophilicity, since lipophilicity is the factor that primarily determines the rate of permeation of substances with similar molecular structure through lipid bilayers [9]. The ratios of the rate of permeation/octanol partition coefficient (Z_{oct}) for the various sugars were very similar (Table II) and comparable to those ob-

TABLE II

RATES OF UPTAKE OF VARIOUS SUGARS BY PIG ERYTHROCYTES AND OF THE OCTANOL PARTITION COEFFICIENTS OF THE SUGARS AT 25 °C

For measuring the uptake of the various sugars, samples of a suspension of $6 \cdot 10^8$ pig erythrocytes/ml were supplemented with 1 mM D-[^{14}C]glucose, L-[^{3}H]glucose, D-[^{3}H]galactose, D-[^{14}C]ribose or D-[^{14}C]xylose. At 1, 5, 10, 20, 40 and 60 min 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. The initial velocities of uptake were estimated graphically from the uptake curves. The first order rate constants (k) were calculated as $k = v/S$. The octanol partition coefficients for the various sugars were determined as described previously [50] with the same radiolabeled substrates used in the uptake experiments.

Sugar	Z_{oct}	k (min^{-1})	k/Z_{oct}
D-Glucose	0.00301 ± 0.00002	0.0030	1.0
L-Glucose	0.00254 ± 0.0013	0.0016	0.64
D-Galactose	0.00810 ± 0.0010	0.0060	0.76
D-Ribose	0.00964 ± 0.00061	0.0075	0.78
D-Xylose	0.00546 ± 0.00047	0.0050	0.91

served for the non-mediated permeation of L-glucose and other substances into other types of mammalian cells [1,50,51]. The results indicate that the pig erythrocytes are deficient in the transport of D-ribose as well as D-glucose. Transport of sugars via the nucleoside transport system was also ruled out by the finding that D-ribose, D-glucose and 3-*O*-methyl-D-glucose, like L-glucose, at concentrations of 100 mM had no significant effect on the equilibrium exchange of 500 μM uridine in pig erythrocytes (data not shown), just as is the case for human erythrocytes [46].

Photoaffinity labeling of nucleoside transporter and identification in stripped membranes

Fig. 5 shows that the protein composition of stripped membranes from human erythrocytes (lane 2) and pig erythrocytes (lane 3) was very similar. On the basis of such SDS-PAGE analyses it has previously been concluded that pig erythrocytes membranes contain over 50% less band 4.5 proteins than human erythrocyte membranes [52], which supported the view that pig red cells lack the carrier protein. We find the levels of proteins in the band 4.5 region (45–60 kDa) of these gels too low and diffuse to allow accurate quantitation.

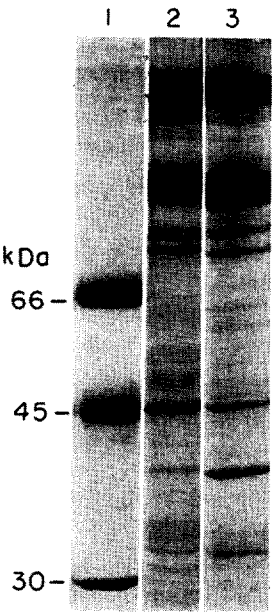

Fig. 5. SDS-PAGE of membrane proteins from human and pig erythrocytes. Stripped membranes from human and pig red cells were analyzed by SDS-PAGE and the gels were stained with Coomassie blue (lanes 2, and 3, respectively). Lane 1 shows appropriate molecular weight standards.

However, gross examination of the gels does not indicate such large differences in total band 4.5 protein levels. Furthermore, when the pig cell membranes were solubilized in Triton X-100, and the solubilized proteins were passed through a DEAE-cellulose column, 12.4% of the total protein was recovered in the flow-through fraction (Fig. 6A). The average recovery in the flow-through fraction in four experiments was $14.7 \pm 1.5\%$ of the total membrane protein, which was comparable to that from analogous human erythrocyte membrane preparations reported by other investigators [21,53] and confirmed in our laboratory ($12.7 \pm 1.2\%$; $n = 12$). A great proportion of the protein in the flow-through fraction represented band 4.5 protein (Fig. 6B). Several bands were detected; the two major ones corresponded to those apparent in SDS-PAGE profiles of total membrane proteins (Fig. 5, lane 3). In addition some low molecular weight protein (20–24 kDa) was present. This protein has not been identified, but similar proteins are often recovered in the band 4.5 fraction of human red cell membranes [21,53].

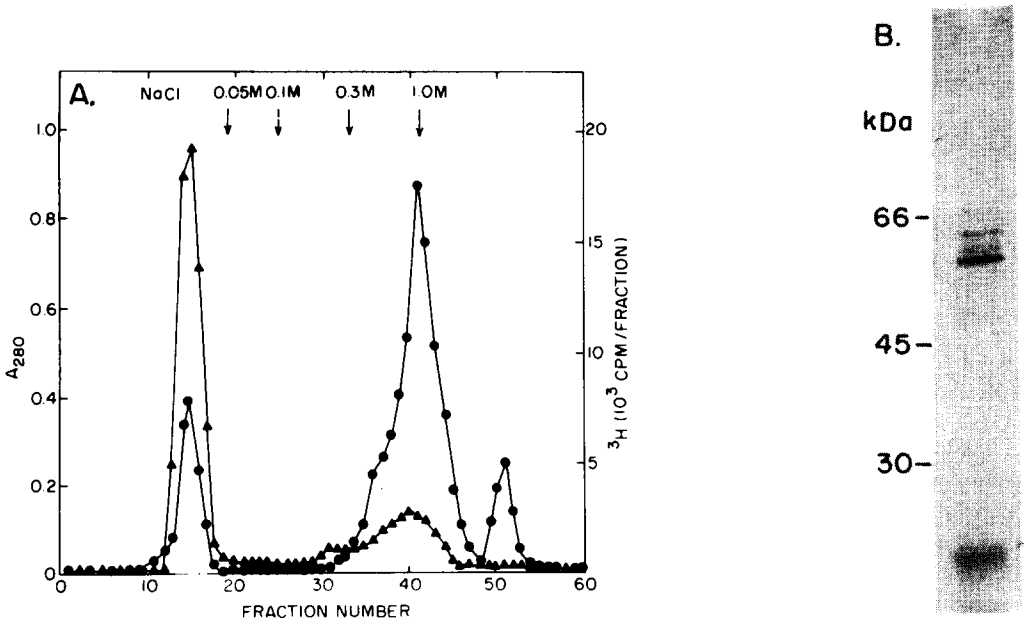

Fig. 6. DEAE-cellulose chromatography of [^{3}H]NBTI-photoaffinity-labeled Triton-solubilized stripped membranes of pig erythrocytes (A) and SDS-PAGE of flow-through proteins (B). Stripped membranes were photoaffinity labelled with [^{3}H]NBTI as described under Experimental procedures, solubilized in 1% Triton X-100 and passed through a DEAE-cellulose column. Fractions from the column were assayed for protein concentrations (absorbance at 280 nm, ●———●) and radioactivity (▲———▲). (B) Fractions 12 to 18 (flow through) were pooled, concentrated and then analyzed by SDS-PAGE using 10% polyacrylamide gels. The gel was silverstained.

Since the band 4.5 proteins of human erythrocytes have been estimated to represent to at least 80% the sugar transporter [22,53–55], our results raise the possibility that membranes from adult pig erythrocytes may possess the sugar transporter in an inactive or cryptic form. Consistent with this view is our preliminary finding that a polyclonal antiserum to human erythrocytes band 4.5 proteins, which is highly specific for this protein fraction (Woffendin et al.; submitted for publication) crossreacted with pig band 4.5 proteins in Western blots (data not shown). However, other factors could account for these results. Antisera to human band 4.5 proteins clearly contain antibodies to the sugar transporter, since such antisera have been successfully used to identify expression vectors carrying cDNAs coding for the sugar transporter in human [56] and rat [57] cDNA libraries, but they probably contain antibodies to other band 4.5 proteins, such as other transporters or perhaps degradation products of other proteins. That the pig band 4.5 proteins represent largely degradation produces of other proteins, however, seems unlikely, since we isolated the band 4.5 proteins from both human and pig erythrocyte membranes in an identical manner in the presence of protease inhibitors.

Little of the solubilized pig membrane proteins not recovered in the flow-through fraction of the DEAE-cellulose column was eluted by 0.1 M NaCl but most was eluted by 0.3 M NaCl (Fig. 6A). Most of the [^{3}H]NBTI-photoaffinity labeled Triton X-100 solubilized protein of pig erythrocytes was recovered in the flow-through fraction of the DEAE-cellulose column (Fig. 6A). The remaining labeled protein was eluted along with most of total protein by 0.3 M NaCl. The high-affinity NBTI binding activity of solubilized pig erythrocyte membranes eluted in the same pattern as the photoaffinity-labeled proteins; > 80% of the activity was recovered in the flow-through fraction of the DEAE-cellulose column (data not shown). Because of the recovery of similar amounts of total protein in this fraction, the specific NBTI-

binding activity of this fraction from pig and human erythrocytes was similar (about 100 pmol/mg protein). This specific binding activity is only slightly higher than that of the stripped membranes due to significant losses of total NBTI binding activity after detergent solubilization, which we attribute to a relatively high instability of the NBTI-binding activity of the solubilized transporter (Woffendin et al., submitted for publication).

Our recovery of the solubilized pig nucleoside transporter in the flow-through fraction of the DEAE-cellulose column clearly differs from the finding reported by Kwong et al. [30,31] that it adsorbs to DEAE-cellulose and is eluted only by 0.2 M NaCl. We have no explanation for this discrepancy. It could be related to the nature of the detergent used to solubilize the band 4.5 proteins, Triton X-100 in our studies and octyl glucoside in the studies by Kwong et al. [30,31]. However, in preliminary experiments we found that most of the pig nucleoside transporter did not bind to DEAE-cellulose whether solubilized in Triton X-100 or octyl glucoside.

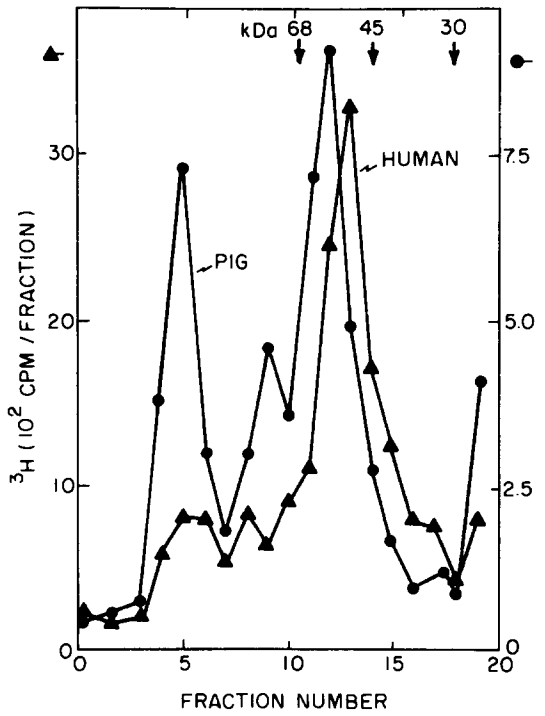

Fig. 7. Photoaffinity labeling of the nucleoside transporters of human (▲———▲) and pig (●———●) erythrocytes. Stripped membranes from human and pig erythrocytes were photoaffinity-labeled with [^{3}H]NBTI and then directly analyzed by SDS-PAGE. The gels were cut into 0.5-cm segments, which were incubated in 30% (v/v) H_2O_2 and analyzed for radioactivity.

Finally, Fig. 7 illustrates SDS-PAGE profiles of [^{3}H]NBTI photoaffinity-labeled proteins from human and pig red cell membranes. Similar 50–65 kDa proteins became photoaffinity labeled in both membranes, but those from pig membranes exhibited a slightly higher apparent molecular weight than those from human erythrocyte membranes. These results are in agreement with an earlier report [15]. We also recovered a considerable amount of radioactivity in a higher molecular weight fraction from pig erythrocyte membranes (Fig. 7), but a similar fraction is regularly photoaffinity-labeled to varying extents in human red cell membranes (Ref. 15, and Woffendin et al. (submitted for publication)). Its presence has been attributed to aggregation of the nucleoside transporter [15,30].

Conclusion

Our results indicate that the nucleoside transporters of human and pig erythrocytes have, within experimental errors, identical kinetic properties and sensitivities to inhibition by NBTI and dipyridamole. They are also both recovered in the band 4.5 protein fraction and after solubilization of stripped membranes in 1% Triton X-100 do not bind to DEAE-cellulose. Thus, at least in these properties, the two transporters are closely related. The main observed difference between them is a slightly higher molecular weight of the pig than the human nucleoside transporter. This difference has recently been reported to be retained after treatment of the solubilized transporters with endoglycosidase F, the treated human and pig transporters exhibiting apparent molecular weights of 45 and 57 kDa, respectively [31]. Furthermore, the oligosaccharides of the pig nucleoside transporter seem resistant to endo-β-galactosidase digestion, whereas those of the human transporter are removed [31]. This apparent difference in oligosaccharide structure has been suggested to possibly account for the reported binding of the octyl glucoside-solubilized pig transporter to

DEAE-cellulose, which has allowed its separation from the bulk of the band 4.5 proteins [31]. These proteins are present in erythrocyte membranes from adult pigs at a similar concentration as in human erythrocyte membranes, which raises the possibility that the pig erythrocytes may possess an inactive or cryptic sugar transporter.

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