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Interaction of [^{3}H]Dipyridamole with the Nucleoside Transporters of Human Erythrocytes and Cultured Animal Cells

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Summary. Equilibrium binding of [^{3}H]dipyridamole identified high-affinity ($K_d \sim 10$ nM) binding sites on human erythrocytes ($\sim 5 \times 10^5$ sites/cell) and on HeLa cells ($\sim 5 \times 10^6$ sites/cell). The equilibration of dipyridamole with these sites on human erythrocytes was compatible with a second-order process which proceeded at 22°C with a rate constant of about 6×10^6 M^{-1} sec^{-1}. Binding of dipyridamole to these sites correlated kinetically with the inhibition of the equilibrium exchange of 500 μM uridine in these cells and was inhibited in a concentration-dependent manner by nucleosides and other inhibitors of nucleoside transport, such as nitrobenzylthioinosine, dilazep and lidoflazine, but not by hypoxanthine, which is not a substrate for the nucleoside transporter of human erythrocytes. The results indicate that the substrate binding site of the transporter is part of the high-affinity dipyridamole binding site. Bound [^{3}H]dipyridamole became displaced from these sites on human erythrocytes by incubation with an excess of unlabeled dipyridamole or high concentrations of nucleosides and inhibitors of nucleoside transport, but neither by hypoxanthine nor sugars. Dissociation of [^{3}H]dipyridamole behaved as a simple first-order process, but the rate constant was about one order of magnitude lower (about 3×10^{-3} sec^{-1}) than anticipated for typical ligand-protein binding on the basis of the measured association rate and equilibrium constants. The reason for this discrepancy has not been resolved. No high-affinity dipyridamole binding sites were detected on Novikoff rat hepatoma cells, P388, L1210 and S49 mouse leukemia cells or Chinese hamster ovary cells, and their absence correlated with a greater resistance of nucleoside transport in these cells to inhibition by dipyridamole. All cells expressed considerable low affinity ($K_d > 0.5$ μM) and nonspecific binding of dipyridamole.

Key Words transport · nucleosides · dipyridamole · human erythrocytes

Introduction

Dipyridamole is a well-known coronary vasolidator and it also affects other metabolic processes in the brain, kidney and platelets (Berne, Rall & Rubio, 1983). Its function in the regulation of blood flow and other metabolic processes is not entirely understood. It is probably indirect and mediated through an inhibition of adenosine transport, since other inhibitors of nucleoside transport, such as dilazep, hexobendine and lidoflazine, exert similar effects as dipyridamole. Adenosine seems to be the primary regulatory factor, whose action is mediated through an interaction with adenyl cyclase-coupled receptors that are present on a variety of cells. The inhibition of adenosine transport is thought to lead to localized increases of free adenosine in tissues, thereby potentiating its receptor interaction.

Dipyridamole also enhances the toxicity of various inhibitors of purine or pyrimidine nucleotide synthesis (Zhen, Lui & Weber, 1983; Cabral et al., 1984; Kang & Kimball, 1984; Nelson & Drake, 1984; Grem & Fisher, 1986) in most instances probably by inhibiting the salvage of extracellular nucleosides by the cell. On the other hand, at concentrations above 10 μM, dipyridamole itself is toxic to a variety of cultured cell lines (Zhen et al., 1983; Plagemann & Wohlhueter, 1985*b*; Plagemann & Kraupp, 1986).

The potent inhibition of nucleoside transport by dipyridamole can be readily demonstrated in studies with erythrocytes and various animal cell cultures (Plagemann & Wohlhueter, 1980; Young & Jarvis, 1983). For example, the zero-*trans* influx of 500 μM uridine in various cultured cell lines is inhibited 50% by concentrations (IC_{50}) of dipyridamole between 50 and 1000 nM (Plagemann & Wohlhueter, 1984*a*). The mechanism of transport inhibition is not understood, but all available evidence indicates that it is more complex than a simple competition with nucleosides for binding to the transporter, even though the kinetics of inhibition have been reported to be strictly competitive (Eilam & Cabantchik, 1977; Jarvis, McBride & Young, 1982) or of mixed type with a primary effect on the Michaelis-Menten constant (Plagemann & Wohlhueter, 1980). In the present study, we have measured directly the binding of [^{3}H]dipyridamole to human erythrocytes and a number of cell lines and have compared its

binding to high-affinity sites with the inhibition of uridine transport.

The nucleoside transporter that is generally expressed by mammalian cells is a nonconcentrative, simple carrier with broad substrate specificity (Plagemann & Wohlhueter, 1980; Young & Jarvis, 1983). The carriers of human erythrocytes and cultured cells exhibit directional symmetry, but differ with respect to mobility of nucleoside-loaded and empty carrier. The loaded carrier of human erythrocytes moves considerably faster than the empty carrier, whereas the transporter of cultured cells does not exhibit any differential mobility of loaded and empty carrier (Plagemann & Wohlhueter, 1980; Plagemann, Wohlhueter & Erbe, 1982; Wohlhueter & Plagemann, 1982; Jarvis et al., 1983*a*). Sensitivity to inhibition by nitrobenzylthioinosine (NBTI) distinguishes two forms of the transporter (Belt, 1983; Belt & Noel, 1985; Plagemann & Wohlhueter, 1984*a*; 1985*a*). One form is inhibited by nanamolar concentrations of NBTI (designated NBTI-sensitive) and the inhibition correlates with binding of NBTI to high-affinity sites (K_d = 0.1 to 1 nM). The other form lacks these sites and is inhibited only at micromolar concentrations of NBTI (designated NBTI-resistant). Some types of cells, such as human erythrocytes, express only NBTI-sensitive transport, whereas some cell lines, such as Novikoff rat hepatoma cells, express only NBTI-resistant transport, and the majority of cells express a combination of the two. Dipyridamole has been reported to competitively inhibit the binding of NBTI to human erythrocytes (K_i = 5 nM; Hammond, Paterson & Clanachan, 1981), HeLa cells (K_i = 30 nM; Paterson et al., 1980) and Chinese hamster ovary (CHO) cells (K_i = 170 nM; Wohlhueter, Brown & Plagemann, 1983), but later studies have shown that the inhibition is not strictly competitive at higher dipyridamole concentrations (Plagemann & Wohlhueter, 1985*a*). The transporter of human erythrocytes has been identified by photoaffinity labeling with [3H]NBTI (Wu et al., 1983) and reconstitution experiments (Tse et al., 1985) as a 45 to 60 kilodalton, band 4.5 membrane glycoprotein.

Materials and Methods

Cells

Erythrocytes from freshly drawn human blood were kindly supplied by Dr. J. Kersey (Department of Pathology, University of Minnesota) as a byproduct of lymphocyte isolation. The cells were thrice washed in cold saline containing 5 mM Tris-HCl (Tris-saline) and suspended in the same or balanced salt solution (BSS) to about 6×10^8 to 1×10^9 cells/ml. Outdated blood was obtained from the blood bank. HeLa, L1210, P388, S49, CHO and Novikoff cells were propagated in suspension culture as described previously (Plagemann & Wohlhueter, 1982; 1983). The cells were harvested from late exponential phase cultures and suspended in BSS at 3×10^6 to 3×10^7 cells/ml. The cells were enumerated with a Coulter counter.

Measurements of Uridine Equilibrium Exchange

The equilibrium exchange of uridine was measured by rapid kinetic techniques as described previously (Wohlhueter et al., 1978; Plagemann & Wohlhueter, 1980; Plagemann et al., 1982). The cells were preincubated with specified concentrations of uridine at 37°C for 40 to 60 min. The suspensions were cooled to 25°C and then the time course of transmembrane equilibration of [3H]uridine at the same concentration as that used for preloading was measured. Samples of cell suspensions were mixed at timed intervals (15 time points per time course) with a solution of [3H]uridine in a ratio of 7.3 : 1 with a dual syringe apparatus, and the cells were separated from the medium by centrifugation through an oil layer in an Eppendorf microfuge. An integrated rate equation based on the simple carrier model (Plagemann & Wohlhueter, 1980; Stein, 1986) was fitted to the time courses. In experiments designed to determine the Michaelis-Menten parameters, six uridine concentrations (80, 160, 320, 640, 1280, 2560 μM) were assayed and the kinetic parameters were extracted by least-squares regression of the pooled data. In other experiments, where only velocities at 500 μM uridine were of interest, the integrated rate equation was fitted with K^{ee}, the Michaelis-Menten constant for equilibrium exchange, fixed at 600 μM (*see* Table 3), and the slope at $t = 0$ was taken as the velocity of exchange (v^{ee}).

Preparation of Stripped Membranes

White ghosts were prepared from human erythrocytes of outdated blood and stripped of peripheral proteins by treatment with 0.1 mM EDTA (pH 11) containing 2.5 mM phenylmethylsulfonyl fluoride and 2.5 mM dithiothreitol as described by Wheeler and Hinkle (1981). The membranes were washed in 50 mM Tris-HCl (pH 7.5) and suspended in the same.

Equilibrium Binding and Dissociation of Dipyridamole

Time courses of binding of [3H]dipyridamole to intact cells were measured by the rapid kinetic technique described already for transport measurements. After separation of the cells from the medium by centrifugation through oil, samples of the supernatant were analyzed for radioactivity (= free radioactivity). Corresponding mixtures of [3H]dipyridamole and BSS were similarly analyzed for total radioactivity. The difference between total and free radioactivity represents that bound by the cells. To measure bound dipyridamole directly, in some experiments (*see* Fig. 1), the cell pellets were also analyzed for radioactivity. After removal of the supernatant, the centrifuge tubes were filled with water, which was then removed along with most of the oil. The cell pellets were suspended in 1 ml of 0.2 N trichloroacetic acid.

The denatured hemoglobin was removed by centrifugation and the clear supernatant fluid was analyzed for radioactivity.

The equilibrium binding of [3H]dipyridamole was measured as previously described for [3H]NBTI (Wohlhueter et al., 1983). Samples of suspensions of cells were mixed in 1.6-ml Eppendorf tubes with radiolabeled and unlabeled dipyridamole to the concentrations indicated in appropriate experiments. After 10 to 20 min of incubation, the suspensions were centrifuged and duplicate samples of the supernatant were analyzed for radioactivity (= free radioactivity). Binding of [3H]NBTI and [3H]dipyridamole to stripped membranes was measured in the same manner, except that the reaction mixtures were cleared of membrane material by centrifugation at 75,000 × *g* for 30 min. Total radioactivity was measured as described already. Radioactivity values were converted to concentrations of total and free dipyridamole (L_f) and the concentration of bound dipyridamole (L_b) was calculated as the difference between total and free. In determinations of the kinetic parameters of equilibrium binding, 12 to 14 concentrations of dipyridamole ranging from 0.2 to 1280 nM were analyzed and the following equation was fitted to the data:

$$L_b = \frac{NL_f}{K_d + L_f} + k'L_f \qquad (1)$$

where N = number of binding sites per liter, K_d = dissociation constant and k' = a coefficient of nonsaturable binding. The equation corresponds to a single saturable site plus a nonspecific component.

For measuring the dissociation of [3H]dipyridamole from the cells, the cells were preincubated with 2.5 or 5 nM [3H]dipyridamole for 5 to 10 min at 22°C. Then samples of the cell suspension were mixed with solutions of unlabeled dipyridamole or of other substances as indicated in appropriate experiments. The suspensions were further incubated at 22 or 25°C and at the indicated times, cleared of cells by centrifugation, and the supernatant was analyzed for radioactivity.

Photoaffinity Labeling of Erythrocyte Membranes

Stripped membranes suspended in 50 mM Tris-HCl (pH 7.4) containing 10 mM dithiothreitol were photoaffinity-labeled in a manner similar to that described by Wu et al. (1983) for [3H]NBTI. The membrane suspensions were supplemented with 10 nM [3H] dipyridamole or 10 nM [3H]NBTI. After about 20 min of incubation at room temperature, the suspensions were placed on ice, and with continuous stirring, irradiated with a germicidal lamp (MR-4: short ultraviolet range, G.W. Gates & Co., Long Island, N.Y.) at a distance of 4 cm for 2 min. The membranes were washed 3 times in 10 mM Tris-HCl (pH 7.4) containing 10 μM dipyridamole or 10 μM NBTI, respectively, and then analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). After electrophoresis, the gels were cut into 0.5-cm segments at a right angle to the direction of migration and the segments were dissolved in 30% H_2O_2 and analyzed for radioactivity.

Materials

[3H-5]uridine, [3H-piperidyl]dipyridamole (Persantin) and [3H] NBTI were purchased from Moravek Biochemicals (Brea, Calif.). Unlabeled nucleosides, hypoxanthine and lidoflazine, were obtained from Sigma (St. Louis, Mo.) and unlabeled NBTI from Calbiochem (San Diego, Calif.) Dipyridamole (Persantin) and dilazep were gifts from Geigy Pharmaceuticals (Yonkers, N.Y.) and Asta Werke AG (Frankfurt, Germany), respectively.

The purity of [3H]dipyridamole was assessed by paper chromatography with solvent 28 (Plagemann & Wohlhueter, 1983) and HPLC on a BioRad reverse phase Hi-Pore RP304 column (250 mm × 4.6 mm) using a Beckman HPLC system with a Hewlett Packard 1040A detection system. The column was eluted in 0.1% trifluoroacetic acid with a 10 to 60% gradient of acetonitrile (the flow rate equaled 1 ml/min) at room temperature.

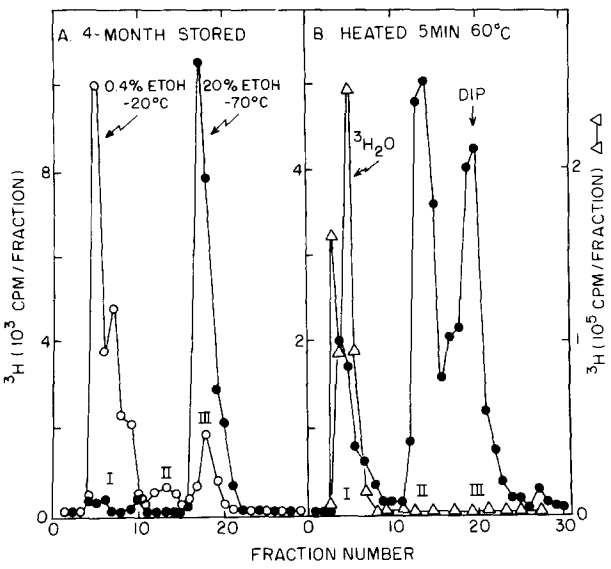

Fig. 1. HPLC analyses of stored and heat-treated [3H]dipyridamole solutions. (*A*) Samples of stock solution of [3H]dipyridamole (500 pCi/ml; 5 μM) in ethanol were diluted 1 : 5 (1 μM, 20% ethanol) and 1 : 250 (20 nM; 0.4% ethanol) in BSS and stored at −70 and −20°C, respectively. After 4 months, samples of the solution were analyzed by HPLC as described under Materials and Methods. (*B*) A sample of the 1 μM [3H]dipyridamole solution was supplemented with 1 N NaOH to a pH of about 11 and heated at 60°C for 5 min before HPLC. A sample of ^3H_2O was chromatographed in the same manner

Results and Discussion

The [3H]dipyridamole, when received from the supplier, was >95% pure when analyzed by paper chromatography and HPLC. The [3H]dipyridamole was relatively stable when stored as a 1-μM (100 μCi/ml) solution in 20% (vol/vol) ethanol at −70°C for 4 months (Fig. 1*A*). About 90% of the radioactivity was still recovered in a fraction (III) that comigrated with authentic unlabeled dipyridamole. However, after a 4-month storage at a concentration of 20 nM (2 μCi/ml) in 0.4% ethanol, about 90% of the radioactivity had been lost from dipyridamole (Fig.

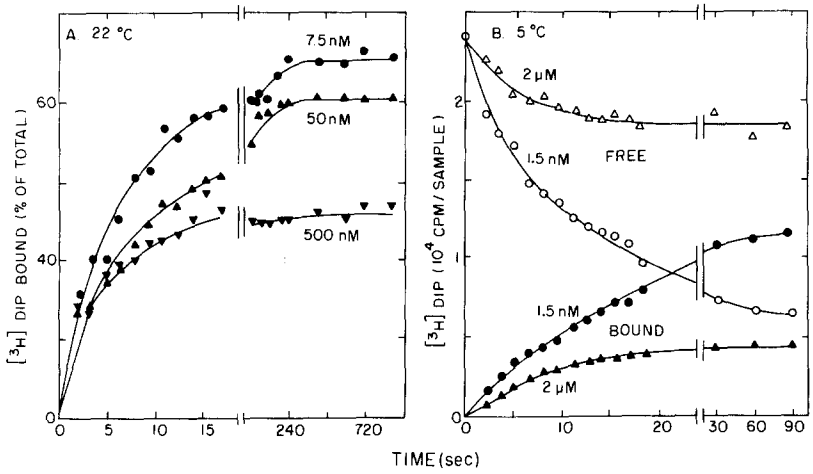

Fig. 2. Time courses of binding of [³H] dipyridamole to human erythrocytes at 22°C (*A*) and 5°C (*B*). The binding of the indicated concentrations of [³H] dipyridamole (DIP) was measured at the two temperatures by rapid kinetic techniques in samples of a suspension of 1 × 10^9 erythrocytes/ml as described under Materials and Methods. The values represent the radioactivity in the medium after removal of the cells by centrifugation (free [³H]dipyridamole) and that associated with the cells (bound [³H]dipyridamole)

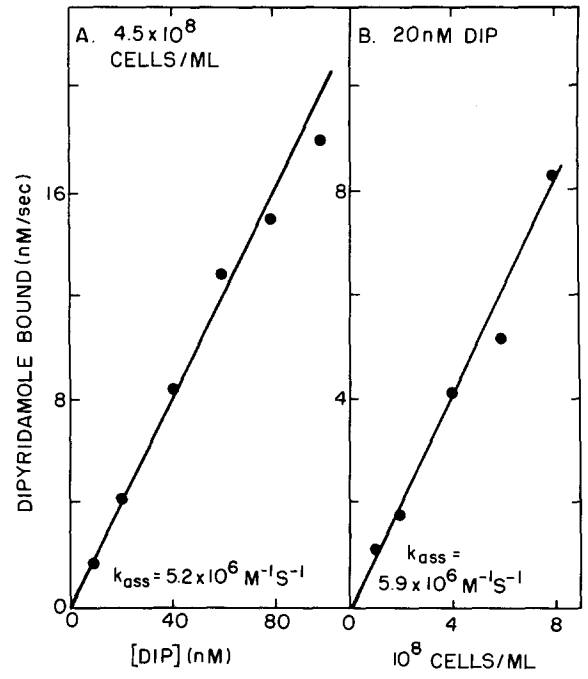

Fig. 3. Reaction order of dipyridamole association with human erythrocytes. The rates of association of [³H]dipyridamole (DIP) with the cells was measured at 22°C by rapid kinetic techniques as described in the legend to Fig. 2. In (*A*), the cell concentration was held constant and the [³H]dipyridamole concentration was varied by addition of labeled dipyridamole as indicated on the abscissa. In (*B*), the [³H]dipyridamole concentration was held constant and the cell density was varied. Second-order rate constants were calculated from the slopes of the lines and the number of high-affinity binding sites per cell (*see* Table 1)

1*A*), and very little binding of radioactivity to cells was observed (*data not shown*). Most of the radioactivity was recovered in a peak (I, encompassing fractions 4 to 10) and a smaller peak (II, fractions 11–14). The degradation products have not been identified. Peak I material co-eluted with ^3H_2O (Fig. 1*B*), but less than 10% of it was volatile (*data not shown*) and thus distinct from ^3H_2O. Heating of the 1-μM solution of [³H]dipyridamole in 20% ethanol at 80°C for about 30 min resulted in the formation of the same radiolabeled components with recovery of 19 and 10% of the radioactivity in peaks I and III, respectively (*data not shown*), and degradation was greatly accelerated under alkaline conditions (Fig. 1*B*). The instability of [³H]dipyridamole seems to be limited to the radiolabel, since we have stored acidic solutions of 2 μM to 2-mM unlabeled dipyridamole for a year and longer without significant alteration in its chromatographic behavior or loss in inhibitory activity for uridine transport (*data not shown*). The [³H]dipyridamole used in all reported experiments was stored at −70°C as a 1-μM stock solution in 20% ethanol and was used within a few hours after dilution to appropriate concentrations.

Figure 2(*A*) illustrates time courses of binding of [³H]dipyridamole to human erythrocytes at various concentrations of dipyridamole (the concentration of [³H]dipyridamole was held constant and its specific activity changed by addition of unlabeled dipyridamole). As expected, equilibration of [³H]dipyridamole at a low nanomolar concentration was relatively slow requiring about 20 seconds for completion. For measuring rates of association we have fitted a smooth curve to the progress curves and estimated the initial rate of association as the slope of the curves at $t = 0$. The rates of association were directly proportional to the dipyridamole concentration in the 10 to 100 nanomolar range (Fig. 3*A*) as well as to the cell density between 1 × 10^8 and 8 × 10^8 cells/ml (Fig. 3*B*), as expected for a second-order reaction. On the basis of 50,000 dipyridamole binding sites/cell (*see later*) we have calculated a second-order rate constant (at 22°C) of 5.5 × 10^6 M^{-1} sec^{-1} (k_{ass}) from the data in Fig. 3.

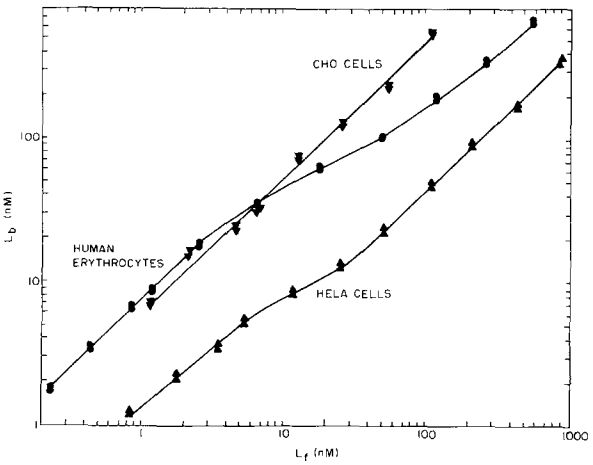

Fig. 4. Amounts of [³H]dipyridamole bound (L_b) at equilibrium to human erythrocytes, HeLa cells and CHO cells as a function of concentration of free dipyridamole (L_f). The equilibrium binding of [³H]dipyridamole at concentrations ranging from 2 to 1280 nM was measured as described under Materials and Methods. The final densities were 7.5 × 10⁸ erythrocytes, 3 × 10⁶ HeLa cells, and 1.4 × 10⁷ CHO cells/ml of BSS

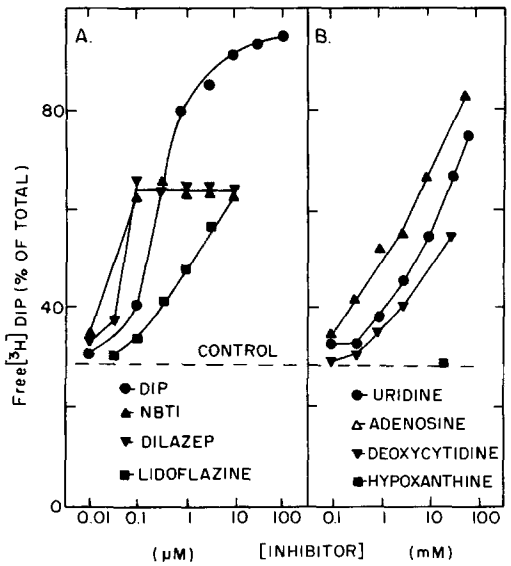

Fig. 5. Effects of nucleosides and nucleoside transport inhibitors on the equilibrium binding of dipyridamole by human erythrocytes. Samples of a suspension of 7.5 × 10⁸ erythrocytes/ml were supplemented with 2.5 nM [³H]dipyridamole (120 cpm/μl) and with the indicated concentrations of unlabeled dipyridamole (DIP), NBTI, dilazep, lidoflazine, uridine, adenosine, deoxycytidine and hypoxanthine. After 10 min of incubation at 22°C, the cells were collected by centrifugation and the supernatant analyzed for radioactivity (free [³H]dipyridamole). All values are averages of duplicate samples. The broken lines indicate the radioactivity remaining in the medium of control cultures, which were incubated with [³H]dipyridamole in absence of any of the test substances

The rate of association of dipyridamole with the cells was only slightly lower at 5 than at 22°C (cf. Fig. 2*A* and *B*). For technical simplicity, we have measured dipyridamole binding to cells in most experiments reported here by the disappearance of [³H]dipyridamole from the extracellular medium (=free dipyridamole). The data in Fig. 1(*B*) demonstrate that the radioactivity recovered in the cell pellet quantitatively correlated with that lost from the medium.

Saturation of dipyridamole binding to human red cells was clearly apparent at both 5 and 22°C (Fig. 2*A* and *B*). Saturation of binding in the nanomolar range is illustrated in more detail in Fig. 4, which presents a log-log plot of the amounts of [³H] dipyridamole bound at equilibrium as a function of the concentration of free dipyridamole. Fitting an equation corresponding to a single saturable binding site plus a linear component for nonspecific binding to the data from this and other similar experiments yielded estimates of the dissociation constant of 4 to 15 nM and of about 50,000 binding sites/human red cell (Table 1). The parameters were similar for erythrocytes from outdated blood and for freshly isolated cells. High-affinity binding of NBTI has also been observed to be similar in fresh and outdated human erythrocytes, even though there is a conspicuous loss of directional symmetry of erythrocyte nucleoside transport during blood storage (Jarvis et al., 1983*a*; Plagemann & Wohlhueter, 1984*b*). The K_d for dipyridamole binding to human erythrocytes was similar to that reported for guinea pig brain membranes (3.5 nM; Marangos, Houston & Montgomery, 1985).

Table 1. Kinetic parameters for the high-affinity binding of dipyridamole to human erythrocytes and HeLa cells[a]

Cells	K_d (nM)	Binding sites/cell	k'
Human erythrocytes, fresh	12.4 ± 2.4	5.8 ± 0.4 × 10⁴	1.0
	4.4 ± 1.3	4.9 ± 0.5 × 10⁴	0.86
	14.9 ± 5.0	5.0 ± 0.6 × 10⁴	0.90
	6.5 ± 0.9	2.3 ± 0.2 × 10⁴	0.85
outdated	19.9 ± 7.1	4.9 ± 0.5 × 10⁴	0.85
HeLa	5.6; 6.0	2 × 10⁶; 3 × 10⁶	

[a] Equilibrium binding of [³H]dipyridamole was measured as described in the legend to Fig. 4 and under Materials and Methods. The values in line 1 are for the data presented in Fig. 4. Equation (1) was fitted to the data for human erythrocytes and the best-fitting parameters ±SE of the estimate are listed. The parameters for HeLa cells were estimated graphically from Scatchard plots of the data in Fig. 4 and of another similar experiment, since fitting Eq. (1) did not yield satisfactory solutions due to relatively high levels of nonspecific or low-affinity binding.

The binding of [³H]dipyridamole to the erythrocytes was further decreased by the presence of con-

Table 2. Equilibrium binding of [3H]dipyridamole in various types of cells[a]

Cells		Cells/ml	[3H]Dipyridamole bound (% of total)			
			10 nM	160 nM	640 nM	10–30 μM
Human erythrocytes	Fresh	7.5×10^8	88	68	54	32
	Outdated	8.0×10^8	84	62	50	32
HeLa		2.6×10^6	58	31	27	25
Novikoff		5.0×10^6	46	43	35	32
P388		2.6×10^7	34	35	33	22
L1210		3.0×10^7	41	42	40	—
CHO		1.4×10^7	35	36	30	30
S49		5.5×10^6	33	31	28	25

[a] The equilibrium binding of 10, 160, 640 and 10,000–30,000 nM [3H]dipyridamole (about 150 cpm/μl, irrespective of concentration) was measured as described under Materials and Methods in suspensions of the various cell types at the cell densities indicated. The values represent the amounts of radioactivity bound (total minus free) as percent of the total radioactivity added.

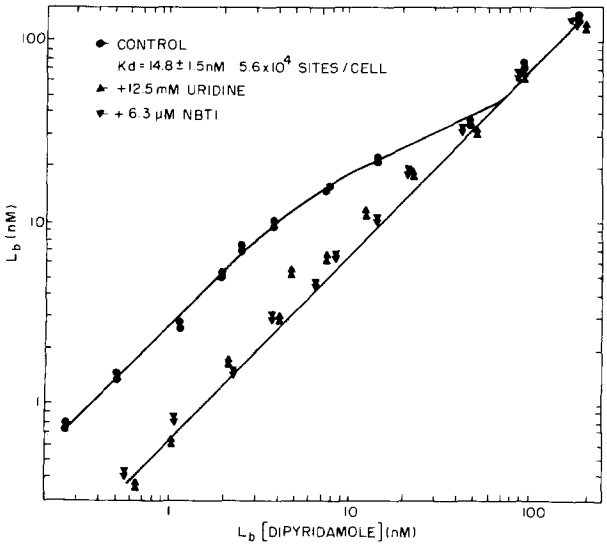

Fig. 6. Effects of uridine and NBTI on equilibrium binding of [3H]dipyridamole. The equilibrium binding of [3H]dipyridamole at concentrations ranging from 1 to 640 nM was measured in samples of a suspension of 5×10^8 red cells/ml in the absence and presence of 12.5 mM uridine and 6.3 μM NBTI as described in Materials and Methods

centrations of unlabeled dipyridamole above that saturating the high-affinity binding sites (Fig. 5*A*; Table 2). This finding is evidence for the presence of additional binding sites with lower affinity, but no distinct low-affinity sites could be identified. The latter were probably of several types, because the apparent saturation was spread over a wide concentration range (Fig. 5*A*; Table 2). In addition, there was considerable nonsaturable binding, which probably included the amounts of [3H]dipyridamole that had equilibrated with the intracellular space and partitioned into membrane lipids. In our experimental design, these amounts could have amounted to 6 to 9% of the total [3H]dipyridamole added. Because of its lipophilicity (*see later*), dipyridamole is expected to very rapidly equilibrate across the plasma membrane.

The dissociation constant for the high-affinity binding of dipyridamole correlated with the concentration of total dipyridamole added (about 20 to 50 nM) that caused a 50% inhibition (IC_{50}) of nucleoside transport in human erythrocytes (Plagemann, 1986; *see later* Fig. 8). This finding suggested that the high-affinity binding is to a site associated with the nucleoside carrier. This conclusion is supported by the following lines of evidence. First, the binding of 2.5 nM [3H]dipyridamole was inhibited by high concentrations of various nucleosides, but not by hypoxanthine (Fig. 5*B*), which is not a substrate for the nucleoside transporter of human erythrocytes (Plagemann & Woffendin, *unpublished data*). Furthermore, the effectiveness of the nucleosides in inhibiting dipyridamole binding correlated with their relative affinity for the nucleoside transporter (adenosine > uridine > deoxycytidine; Plagemann & Wohlhueter, 1980). Dipyridamole binding was also inhibited by other inhibitors of nucleoside transport such as NBTI, dilazep and lidoflazine (Fig. 5*A*), but they maximally reduced the binding of 2.5 nM [3H]dipyridamole less than unlabeled dipyridamole. These inhibitions of dipyridamole binding were probably mainly at the high-affinity sites. As shown in Fig. 6, the presence of NBTI as well as uridine completely inhibited the binding of [3H]dipyridamole to high-affinity sites, while not affecting low-affinity or nonspecific binding.

Second, the time courses of binding of dipyridamole to high-affinity sites correlated with the time course of establishment of inhibition of nucleoside transport (cf. Fig. 2 and Fig. 7*B*). Figure 7 illustrates time courses of equilibrium exchange of 500 μM uridine in suspensions of human erythrocytes, which had been preincubated with various concentrations of dipyridamole before the exchange of [3H] uridine was measured (frame *A*) or which were supplemented with the same concentrations of dipyridamole simultaneously with the radiolabeled uridine (frame *B*). The results show that maximum inhibition of uridine exchange was attained only 3 to 5 sec after addition of <1 μM dipyridamole to the cells. Because of this relatively slow action of dipyridamole at these concentrations and the rapidity

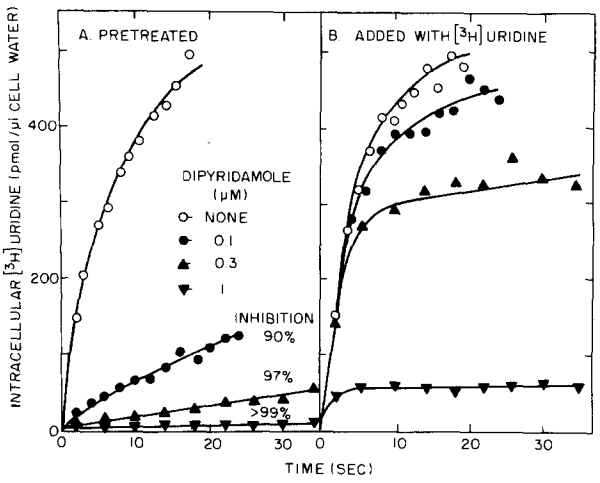

Fig. 7. Effects of dipyridamole on the equilibrium exchange of 500 μM [3H]uridine when added prior to measuring the equilibration of [3H]uridine (*A*) or when added simultaneously with [3H] uridine (*B*). A suspension of 7×10^8 human erythrocytes/ml was equilibrated with 500 μM unlabeled uridine. Then in (*A*), samples of the suspension were supplemented with the indicated concentrations of dipyridamole and, after at least 2 min, the transmembrane equilibration of [3H]uridine was measured by the rapid kinetic techniques described under Materials and Methods. In (*B*), the indicated concentrations of dipyridamole were mixed with the cell suspensions along with the [3H]uridine. In (*A*), the velocities of exchange (v^{ee}) were estimated by integrated rate analysis of the time courses and served for calculating the percent inhibition of exchange

of nucleoside transport in human erythrocytes, [3H] uridine equilibration across the membrane was close to complete before a significant inhibition of carrier function was effected. For example, addition of 100 nM dipyridamole simultaneously with [3H]uridine had relatively little effect on its equilibration (Fig. 7*B*), whereas pretreatment of the cells with the same concentration of dipyridamole inhibited its equilibration about 90% (Fig. 7*A*).

Dipyridamole at concentrations ≤ 1 μM had about the same delayed effect on the efflux of 500 μM [3H]uridine from preloaded cells as on inward equilibrium exchange (*data not shown*), and a similar delayed inhibition of nucleoside transport has been observed for nanomolar concentrations of NBTI and lidoflazine (Mahony & Zimmerman, 1986; Plagemann & Woffendin, *unpublished data*). The relative slowness of action of these inhibitors at lower concentrations needs to be taken into consideration in their use as "stoppers" in nucleoside transport assays (Paterson, Kolassa & Cass, 1981). High enough concentrations of inhibitor need to be used to achieve an inhibition that is "instantaneous" in relation to the rate of nucleoside transport exhibited by the system under investigation.

Table 3. Effects of dipyridamole on the kinetics of uridine exchange in human erythrocytes[a]

Dipyridamole (nM)	K^{ee} (μM)	V^{ee} (μM/sec)
0	644 ± 77	166 ± 7
20	1096 ± 111	127 ± 6
40	2135 ± 339	138 ± 13
80	2614 ± 434	87 ± 9

[a] Portions of a suspension of 6×10^8 erythrocytes/ml were equilibrated with six concentrations of unlabeled uridine (80 to 2560 μM). Then samples of each suspension were supplemented with the indicated concentrations of dipyridamole and the transmembrane equilibration of [3H]uridine (313 cpm/μl, irrespective of concentration) at the same concentration as used in preloading the cells was measured by the rapid kinetic technique described under Materials and Methods. The integrated rate equation for equilibrium exchange was fitted to the pooled data for each inhibitor concentration. The best-fitting parameters ±SE of the estimate are listed.

In human erythrocytes the development of inhibition of nucleoside transport by nanomolar concentrations of dipyridamole and of other tight binding inhibitors is too slow relative to the transport rate to allow assessment of the kinetics of inhibition by conventional analysis in which the cells are exposed simultaneously to substrate and inhibitor. We have, therefore, pretreated the cells with various concentrations of dipyridamole and then determined the kinetics of uridine equilibrium exchange (Table 3). Treatment of the cells with dipyridamole resulted in both an increase in the Michaelis-Menten constant (K^{ee}) and a decrease in maximum velocity (V^{ee}), but the decrease in K^{ee} was the major effect. Plots of K^{ee}/V^{ee} and of $1/V^{ee}$ against the dipyridamole concentration according to Segel (1975) yielded values of $K_{i,\text{slope}}$ and $K_{i,\text{intercept}}$ of 12 and about 100 nM, respectively. It should be emphasized that these values are based on the concentration of total dipyridamole in the suspension and not on the concentration of free inhibitor. Since under the conditions of the experiment, between 60 and 80% of the total dipyridamole was bound to the cells, the $K_{i,\text{slope}}$ and $K_{i,\text{intercept}}$ were about 2.5 and 20 nM, respectively, when expressed on the basis of the concentration of free dipyridamole. Similar values were obtained in a second experiment of this type (*data not shown*). This apparent mixed type of inhibition caused by pretreatment of the cells with dipyridamole was similar to that reported previously for Novikoff rat hepatoma cells, except that the apparent K_i values were considerably lower for the erythrocytes than for Novikoff cells ($K_{i,\text{slope}} \sim 3$ μM and $K_{i,\text{intercept}} \sim 12$ μM; Plagemann & Wohlhue-

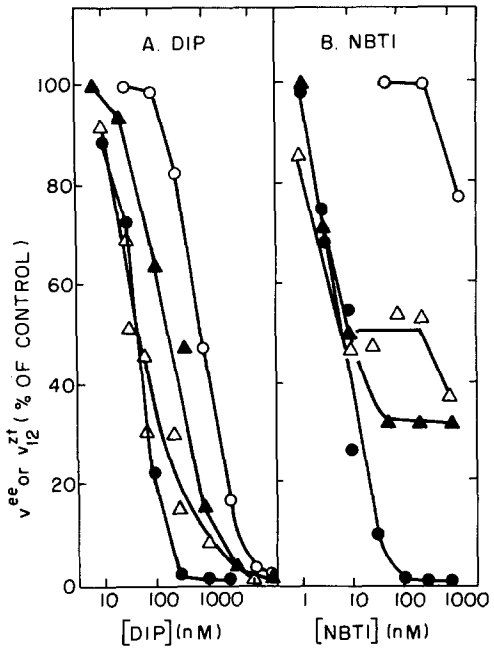

Fig. 8. Velocities of equilibrium exchange of uridine in human erythrocytes (●–●) and of zero-*trans* entry of uridine in Novikoff (○–○), HeLa (△–△) and CHO (▲–▲) cells as a function of the concentrations of dipyridamole (*A*) and NBTI (*B*). The equilibrium exchange of 500 μM uridine by human erythrocytes was measured as illustrated in Fig. 7*A*. Zero-*trans* influx in the cultured cells was measured similarly, except under zero-*trans* conditions. The data for Novikoff and CHO cells are from Plagemann and Wohlhueter (1984*a*) and are shown for comparative purposes. In each case, the cells were pretreated with the indicated concentrations of dipyridamole (DIP) or NBTI before the uptake of [3H]uridine was measured. The velocities of exchange (v^{ee}) and zero-*trans* entry (v_{12}^{zt}) were estimated by integrated rate analysis of the time courses as described under Materials and Methods and by Plagemann and Wohlhueter (1980; 1984*a*,*b*). The velocities are expressed as percent of those of the control cultures not treated with inhibitor

ter, 1980) in accordance with the greater sensitivity of nucleoside transport in human erythrocytes than in Novikoff cells and some other cultured cell lines to inhibition by dipyridamole (Fig. 8*A*; Plagemann & Wohlhueter, 1984*a*). The greater resistance of nucleoside transport in certain cell lines than in human erythrocytes is probably related to a lower affinity of the carrier of these cells for dipyridamole and may explain our failure to detect high-affinity binding of dipyridamole to CHO cells, Novikoff cells, L1210 and P388 mouse leukemia cells, and S49 mouse lymphoma cells (Fig. 4; Table 2). There was only evidence of low-affinity binding of dipyridamole in these cells (Table 2). Only in HeLa cells did we detect some high-affinity binding of dipyridamole similar to that in erythrocytes (Fig. 4, Table 2), which correlated with a similar sensitivity of nucleoside transport to inhibition by dipyridamole in the two types of human cells (Fig. 8*A*). Whereas the sensitivity of nucleoside transport to dipyridamole differed considerably for different types of cells, the sensitivity to inhibition by NBTI of NBTI-sensitive nucleoside transport in various cell types varied much less (cf. Figs. 8*A* and 8*B*; Plagemann & Wohlhueter, 1984*a*).

The third line of evidence indicating that the high-affinity binding of [3H]dipyridamole involves the nucleoside carrier is provided by photoaffinity labeling by [3H]dipyridamole of band 4.5 integral plasma membrane proteins of human erythrocytes (Fig. 9*A*), which also become photoaffinity labeled with [3H]NBTI (Fig. 9*B*) and are believed to represent the nucleoside carrier (Wu et al., 1983). This conclusion is supported by the finding that the photoaffinity labeling with [3H]dipyridamole was inhibited by the presence of uridine and NBTI (Fig. 9*A*) as well as by 1 μM dilazep or 20 mM adenosine (*data not shown*). Similarly, the photoaffinity labeling of this protein with [3H]NBTI is inhibited by dipyridamole and nucleosides (Wu et al., 1983). A photoaffinity labeling with [3H]dipyridamole similar to that shown in Fig. 9*A* was observed in two other experiments (*data not shown*). In each case, proteins with higher molecular weight also became labeled. We have observed the same type of labeling to varying extents with [3H]NBTI (Woffendin & Plagemann, *unpublished data*). Since labeling of the higher molecular weight proteins was also prevented by the presence of nucleosides and NBTI (Fig. 9*A*), it could be argued that these might represent multimers of the 50 to 60 kD carrier monomer. Radiation target size studies have suggested that the nucleoside carrier as well as the sugar transporters in their functional forms in the plasma membrane are probably dimers (Jarvis et al., 1984; Jarvis, Ellory & Young, 1986). On the other hand, in the case of the sugar transporter of human erythrocytes it has been concluded that similar high molecular weight cytochalasin B-binding proteins of the band 4.5 fraction represent denatured carrier (Rampal et al., 1986).

The apparent number of high-affinity dipyridamole binding sites per erythrocyte (about 50,000; Table 1) is considerably higher than the values reported for high-affinity NBTI binding sites (11,000 to 15,000; Cass et al., 1974; Jarvis, Janmohamed & Young, 1983*b*; Plagemann & Wohlhueter, 1984*b*). A similar three-fold difference in the number of dipyridamole and NBTI binding sites has been reported for guinea pig brain membranes (Marangos et al., 1985). Such differences could suggest the presence of additional high-affinity dipyridamole binding sites distinct from the nucleoside carrier. However, this seems unlikely because of the complete inhibi-

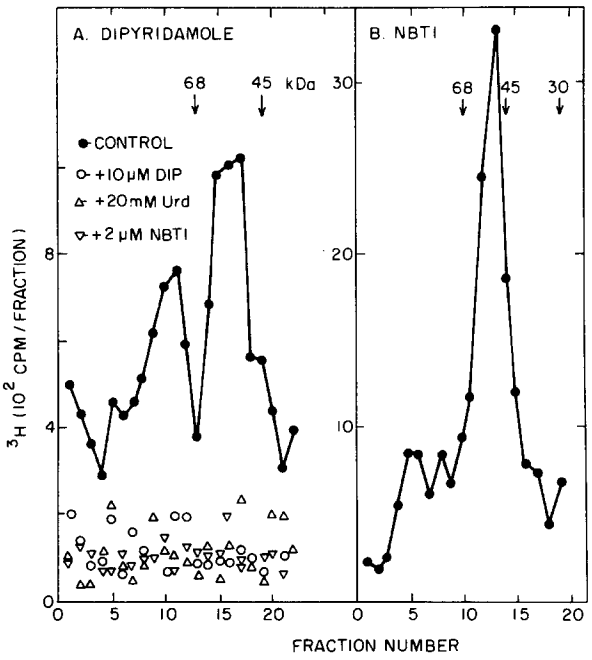

Fig. 9. Photoaffinity labeling of band 4.5 protein of human erythrocyte membranes by [³H]dipyridamole (*A*) and [³H]NBTI (*B*). Stripped membranes (~ 2 mg protein/ml) were equilibrated with 10 nM [³H]dipyridamole (DIP) or 10 nM [³H]NBTI in the presence, where indicated, of 10 μM unlabeled dipyridamole (DIP), 20 mM uridine (Urd) or 2 μM NBTI, then irradiated and analyzed by SDS-PAGE as described under Materials and Methods. The molecular weight standards were: bovine serum albumin (68 kD), ovalbumin (45 kD) and carbonic anhydrase (30 kD)

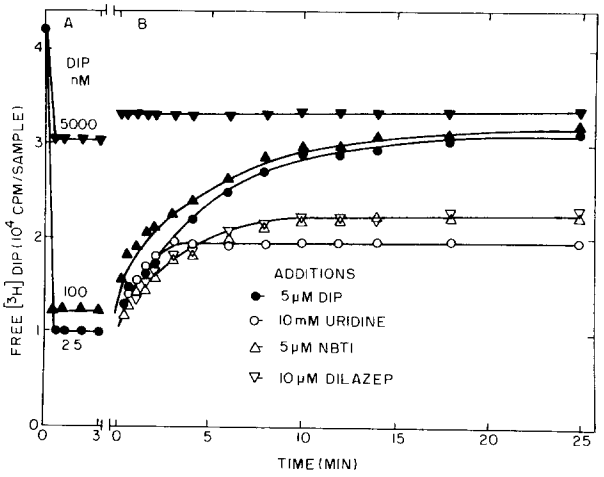

Fig. 10. Time courses of release of [³H]dipyridamole from human erythrocytes in the presence of unlabeled dipyridamole, uridine, NBTI and dilazep. Samples of a suspension of 8 × 10⁸ red cells/ml were supplemented with 2.5 (●–●), 100 (▲–▲) or 5000 nM (▼–▼) [³H]dipyridamole (DIP). (*A*) At 0.5, 1, 2 and 3 min of incubation at 22°C, samples of each suspension were centrifuged and the supernatant analyzed for radioactivity (free [³H]dipyridamole). (*B*) At about 5 min, samples of each of these suspensions were supplemented with 5 μM unlabeled dipyridamole, further incubated at 22°C and monitored for free [³H]dipyridamole as already described. Other samples of the suspension that had been equilibrated with 2.5 nM [³H]dipyridamole were supplemented as indicated with 10 mM uridine, 5 μM NBTI or 10 μM dilazep and also monitored for free [³H]dipyridamole during further incubation

tion of the high-affinity binding of dipyridamole and of the photoaffinity labeling of the band 4.5 proteins by nucleosides and other inhibitors of nucleoside transport (Figs. 6 and 9). Another possibility is that one molecule of nucleoside carrier is associated with three high-affinity dipyridamole binding sites, whereas the ratio is assumed to be 1:1 for NBTI binding (Young & Jarvis, 1983). A third possible explanation is that the number of high-affinity dipyridamole binding sites on whole cells is overestimated due to the relatively high levels of low-affinity and/or nonspecific binding of dipyridamole to the cells (Fig. 2). This conclusion is supported by the finding that the difference in the number of molecules of [³H]dipyridamole and of [³H]NBTI bound was smaller for stripped membranes, which exhibited less nonspecific or low-affinity binding, than intact cells (B_{max} = 93 and 61 pmol/mg protein, respectively, *data not shown*). The same conclusion may apply to the estimated number of dipyridamole binding sites on HeLa cells (2 × 10⁶ sites/cell; Table 1), which compares to 2 × 10⁵ to 4 × 10⁵ NBTI binding sites/HeLa cell reported previously (Lauzon & Paterson, 1977; Cass et al., 1979) and 2× 10⁵ sites/cell (K_d = 0.16 ± 0.11 nM) estimated in the present study for the line of HeLa cells propagated in our laboratory and used in the dipyridamole binding experiment. However, only about 50% of the nucleoside carriers of HeLa cells are in the NBTI-sensitive form (Paterson et al., 1980; Fig. 8*B*), so that the actual number of nucleoside carriers as estimated from the NBTI binding data would be 4 × 10⁵ to 8 × 10⁵/HeLa cell.

We have also measured the dissociation of [³H] dipyridamole from high-affinity sites on human erythrocytes in the presence of an excess of unlabeled dipyridamole (Fig. 10). Dissociation behaved as a simple first-order process, but dissociation was considerably slower than anticipated from the measured rate constant for association (k_{ass}) and the equilibrium constant (K_d). The dissociation constants (k_{diss}) estimated from the data in Fig. 10 and three other similar experiments fell between 0.145 and 0.218 min⁻¹, and were the same whether cells equilibrated with 2.5 or 100 nM [³H]dipyridamole were incubated with 1, 5 or 20 μM unlabeled dipyri-

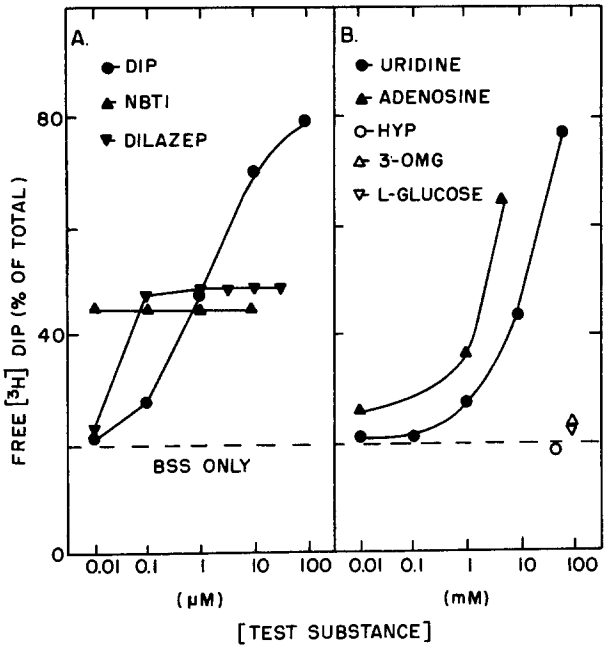

Fig. 11. Displacement of [³H]dipyridamole from human erythrocytes as a function of concentration of unlabeled dipyridamole or other substances. A suspension of 2.5 × 10^9 red cells/ml was equilibrated with 5 mM [³H]dipyridamole at 22°C for 10 min. Then samples of the suspension were diluted 1:4 with BSS or with BSS containing unlabeled dipyridamole (DIP), NBTI, dilazep, uridine, adenosine, hypoxanthine (Hyp), 3-0-methyl-D-glucose (3-OMG) or L-glucose to yield the indicated concentrations. After 30 min of incubation at 22°C, the suspensions were clarified of cells by centrifugation and the supernatants analyzed for radioactivity (free [³H]dipyridamole). All values are expressed as percent of total [³H]dipyridamole (free + bound) and are averages of duplicate samples. The broken lines indicate the radioactivity in the cell-free medium of suspensions diluted with BSS alone

damole (k_{diss} = 0.183 ± 0.01 min^{-1}; n = 7). The measured values for k_{ass} (3.3 × 10^8 M^{-1} min^{-1}) and K_d (10 nM) would have predicted the k_{diss} (= $K_d \cdot k_{ass}$) to be about 3.3 min^{-1}. The reasons for this discrepancy are not known. It could be due to the simultaneous dissociation of [³H]dipyridamole from both high-affinity nucleoside carrier sites and sites with lower affinity. However, the dissociation effected by uridine, NBTI and dilazep, which probably reflected dissociation from the nucleoside carrier, was similarly as slow as that observed in the presence of unlabeled dipyridamole (Fig. 10). The presence of NBTI and dilazep caused the dissociation of a maximum of only 50% of the bound dipyridamole (Fig. 11*A*). Uridine and adenosine were more effective but only at relatively high concentrations (>1 mM). Nevertheless, these effects seemed nucleoside carrier-specific since high concentrations of hypoxanthine or of sugars did not cause a dissociation of dipyridamole. Sugar transport is inhibited by dipyridamole (Deuticke, Duhm & Gerlach, 1964), but the IC_{50} is about 100 times higher than that for the inhibition of nucleoside transport (Plagemann & Woffendin, *unpublished data*), which implies that the K_d for binding of dipyridamole to the sugar transporter falls between 10 to 100 μM rather than in the nanomolar range.

These results and earlier work (Koren, Cass & Paterson, 1983; Wohlhueter et al., 1983; Belt & Noel, 1985; Plagemann & Wohlhueter, 1985*a*) indicate that the interaction of dipyridamole with the carrier is more complex than a competition between dipyridamole and nucleosides for the substrate binding site of the carrier. We have previously suggested that inhibition of nucleoside transport by dipyridamole and other substances structurally unrelated to the carrier substrates, as well as by NBTI, involves an interaction of the lipophilic inhibitors with hydrophobic domains of the carrier or hydrophobic molecules closely associated with the substrate binding site of the transporter (Wohlhueter et al., 1983; Plagemann & Wohlhueter, 1980; 1985*a*). Perhaps this interaction requires partitioning of the inhibitors into and movement within the lipid bilayer of the plasma membrane. Partitioning into the lipid bilayer is expected to be very rapid because of the lipophilicity of the inhibitors, and not to be rate limiting in the binding of the inhibitors to their high-affinity binding sites. The octanol partition coefficient for [³H]NBTI has previously been reported to be about 30 (Jarvis et al., 1982; Wohlhueter et al., 1983). That for [³H]dipyridamole has been determined to be 3.77 ± 0.29.

The binding sites for dipyridamole and NBTI clearly overlap and the nucleoside binding site of the carrier seems to be a common component of both. The alternate view that the binding of the inhibitors or of nucleosides causes conformational alterations in the carrier that inhibit the binding of each other seems less likely. However, several lines of evidence support the view that dipyridamole and NBTI interact with the nucleoside transporter in different ways (Wohlhueter et al., 1983; Belt & Noel, 1985; Plagemann & Wohlhueter, 1985*a*) or in other words, that the high-affinity binding sites for NBTI and dipyridamole are not identical. For example, the two substances do not inhibit the binding of each other in a strictly competitive manner (Plagemann & Wohlhueter, 1985*a*), both NBTI-resistant and sensitive nucleoside transport are inhibited by dipyridamole (Plagemann & Wohlhueter, 1984*a*; Belt & Noel, 1985; *see* Fig. 8) and the sensitivity of the nucleoside transporters of different types of cells to inhibition by NBTI and dipyridamole differ

significantly (Fig. 8; Plagemann & Wohlhueter, 1984*a*). Clearly, further work is required to characterize these binding sites at the molecular level.

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