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Nucleoside Transport in Rat Erythrocytes: Two Components with Differences in Sensitivity to Inhibition by Nitrobenzylthioinosine and *p*-Chloromercuriphenyl Sulfonate

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Summary. The sensitivity of nucleoside transport by rat erythrocytes to inhibition by nitrobenzylthioinosine (NBMPR) and the slowly permeating organomercurial, *p*-chloromercuriphenyl sulfonate (*p*CMBS), was investigated. The dose response curve for the inhibition of uridine transport (100 μM) by NBMPR was biphasic—35% of the transport activity was inhibited with an IC_{50} value of 0.25 nM, but 65% of the activity remained insensitive to concentrations as high as 1 μM. These two components of uridine transport are defined as NBMPR-sensitive and NBMPR-insensitive, respectively. Uridine influx by both components was saturable and conformed to simple Michaelis-Menten kinetics, and was inhibited by other nucleosides. The uridine affinity of the NBMPR-sensitive transport component was threefold higher than for the NBMPR-insensitive transport mechanism (apparent K_m for uridine 50 ± 18 and 163 ± 28 μM, respectively). The two transport systems also differed in their sensitivity to *p*CMBS. NBMPR-insensitive uridine transport was inhibited by *p*CMBS with an IC_{50} of ~25 μM, while 1 mM *p*CMBS had little effect on NBMPR-sensitive transport by intact cells. *p*CMBS inhibition was reduced in the presence of uridine and adenosine and reversed by the addition by β-mercaptoethanol, suggesting that the *p*CMBS-sensitive thiol group is located on the exterior surface of the erythrocyte membrane within the nucleoside binding site of the transport system. Inhibition of uridine transport by NBMPR was associated with high-affinity [^{3}H]NBMPR binding to the cell membrane (apparent K_d 46 ± 25 pM). Binding of inhibitor to these sites was competitively blocked by uridine and inhibited by adenosine, thymidine, dipyridamole, dilazep and nitrobenzylthioguanosine. Assuming that each NBMPR-sensitive transport site binds a single molecule of NBMPR, the calculated translocation capacity of each site is 25 ± 6 molecules/site per sec at 22°C. *p*CMBS had no effect on [^{3}H]NBMPR binding to intact cells but markedly inhibited binding to disrupted membranes indicating that the NBMPR-sensitive nucleoside transporter probably has a thiol group located on the inner surface of the membrane. Exposure of rat erythrocyte membranes to UV light in the presence of [^{3}H]NBMPR resulted in covalent radiolabeling of a membrane protein(s) (apparent M_r on SDS gel electropherograms of 62,000). Labeling of this protein was abolished in the presence of nitrobenzylthioguanosine. We conclude that nucleoside transport by rat erythrocytes occurs by two facilitated-diffusion systems which differ in their sensitivity to inhibition by both NBMPR and *p*CMBS.

Key Words nucleoside transport · nitrobenzylthioinosine · *p*-chloromercuriphenyl sulfonate · thiol · red cell · photoaffinity labeling

Introduction

In many types of cells where nucleoside transport occurs by a facilitated diffusion process, nanomolar concentrations of nitrobenzylthioinosine (NBMPR) inhibit uptake (for review *see* Young & Jarvis, 1983). Inhibition by NBMPR is associated with high-affinity binding of the inhibitor to the cell membrane and occupancy of these binding sites in human erythrocytes is directly proportional to inhibition of nucleoside transport (Cass et al., 1974, 1981; Jarvis & Young, 1980). NBMPR is a competitive inhibitor of uridine influx and equilibrium exchange influx by nucleoside-permeable sheep and human erythrocytes, respectively (Eilam & Cabantchik, 1977; Jarvis et al., 1982*b*). Similarly, nucleosides block high-affinity NBMPR binding in an apparent competitive manner with apparent K_i values close to the apparent K_m values for equilibrium exchange transport (Jarvis et al., 1982*b*; 1983; Hammond & Clanachan, 1984; Wu & Young, 1984). Furthermore, genetic variants of both sheep erythrocytes and S49 mouse lymphoma cells, which do not transport nucleosides by a saturable system, also do not possess high-affinity NBMPR binding sites (Jarvis & Young, 1980; Cass et al, 1981). On the basis of these data, the general view until recently was that the facilitated diffusion of nucleosides across the plasma membrane of mammalian cells was of a single type inhibitable by NBMPR, this inhibition being associated with high-affinity binding of ligand to the transporter.

However, recent studies on cultured animal

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cells have clearly indicated that this proposition is an oversimplification. In some cell lines, such as Novikoff hepatoma cells (Plagemann & Wohlhueter, 1984*a*) and Walker 256 carcinosarcoma cells (Paterson et al., 1983; Belt & Noel, 1985) high concentrations of NBMPR (1 μM) have little effect on saturable nucleoside transport. Consistent with this observation is the finding that these cells also lacked high-affinity sites for NBMPR (Paterson et al., 1983; Plagemann & Wohlhueter, 1984*a*), although such sites are present on cells of another Novikoff hepatoma cell line (Novikoff-UA cells) where nucleoside transport is also not inhibited by nanomolar concentrations of NBMPR (Gati et al., 1986). The molecular properties of these NBMPR binding sites on Novikoff-UA cells are different from those on cells where NBMPR inhibits transport with a high sensitivity. Photoaffinity labeling studies with [^{3}H]NBMPR have demonstrated that the molecular weight of the human erythrocyte and the S49 mouse lymphoma NBMPR binding polypeptide, as revealed by SDS-gel electrophoresis, is 65,000 to 45,000, but 80,000 to 72,000 for Novikoff-UA cells (Wu et al., 1983*a*,*b*; Young et al., 1984; Gati et al., 1986). Thus, the relationship of the NBMPR binding sites on Novikoff-UA cells with the nucleoside transport mechanism is unclear. A further complication is that in some cultured cells, for example methylcholanthrene-transformed hamster embryo cells (MCT), HeLa, L1210 and Chinese hamster ovary (CHO), nucleoside transport is only partially inhibited by nanomolar concentrations of NBMPR (Eilam & Cabantchik, 1977; Bibi et al., 1978; Heichal et al., 1978; Dahlig-Harley et al., 1981; Belt, 1983; Plagemann & Wohlhueter, 1984*a*). These cell lines all possess high-affinity NBMPR binding sites although the molecular properties of these sites have not been investigated (Eilam & Cabantchik, 1977; Dahlig-Harley et al., 1981; Plagemann & Wohlheuter, 1984*a*). Nucleoside transport by the NBMPR-insensitive component in L1210 and CHO cells is saturable with a substrate specificity similar to that of the NBMPR-sensitive component (Belt, 1983; Plagemann & Wohlhueter, 1984*a*).

These results raise the question of whether the different forms of nucleoside transport observed in cultured cells can also be detected in noncultured cells. Since many of the reported differences in nucleoside transport occur in cells of rat origin, we have investigated the sensitivity of nucleoside transport in rat erythrocytes to NBMPR. Nucleoside transport in these cells was resolved into two components. The first component was sensitive to inhibition by nanomolar concentrations of NBMPR but insensitive to extracellular *p*-chloromercuriphenyl sulfonate (*p*CMBS), while the second component of transport was sensitive to extracellular *p*CMBS but had a low sensitivity (μM) to NBMPR inhibition. In contrast to cultured cells, the substrate affinities of the two transport systems also differed. During the preparation of this manuscript, Plagemann and Wohlhueter (1985) reported a similar observation that nucleoside transport by rat erythrocytes was only partially inhibited by NBMPR, but the properties of the two transport components were not investigated in detail.

Materials and Methods

Materials

[U-^{14}C]Uridine (specific radioactivity 529 mCi/mmol) and [G-^{3}H]NBMPR (specific radioactivity 17 Ci/mmol) were obtained from Amersham (Oakville, Ontario) and Moravek Biochemicals (Brea, CA), respectively. NBMPR and NBTGR, and dilazep were generous gifts from Professor A.R.P. Paterson, Cancer Research Group, University of Alberta, Edmonton and Hoffmann-LaRoche and Co., Basle, Switzerland, respectively. Unlabeled nucleosides, dipyridamole and *p*CMBS were purchased from Sigma Chemical Co. (St. Louis, MO). It should be noted that in neutral solution the hydroxyl form *p*CMBS prevails. Gel electrophoresis reagents were obtained from Bio-Rad Laboratories (Misissauga, Ontario).

Erythrocytes and Membranes

Blood from male Sprague-Dawley rats and human volunteers was collected into heparinized tubes and centrifuged at 1,000 × *g* for 5 min. The plasma and buffy coats were discarded, and the erythrocytes were washed three times with 20 volumes of isosmotic NaCl medium containing 140 mM NaCl, 5 mM KCl, 20 mM Tris-HCl (pH 7.4 at 22°C), 2 mM $MgCl_2$, 0.1 mM EDTA (disodium salt), 5 mM glucose and 0.05% (wt/vol) BSA. The washed cells were filtered through cotton wool to remove residual white blood cells and platelets (Beutler et al., 1976). In those experiments where the effect of sulfhydryl reagents on nucleoside transport activity was investigated the EDTA and BSA in the isosmotic NaCl medium were replaced by 2 mM $CaCl_2$. Hemoglobin-free erythrocyte membranes were prepared by the method of Dodge et al. (1963) and suspended in 5 mM sodium phosphate (pH 7.2).

Nucleoside Transport

Initial rates of zero-trans uridine influx at 22°C were determined by a washing method using ice-cold isosmotic NaCl medium containing 20 μM dipyridamole, the details of which have been published previously (Young, 1978; Jarvis et al., 1982*a*,*b*). The washing procedure removes extracellular labeled permeant, without significant loss of intracellular radioactivity from cells with slow transport rates (Young & Ellory, 1982). Briefly, 0.2-ml portions of cell suspension (hematocrit, 20%) were mixed with 0.2 ml of isosmotic NaCl medium containing the appropriate concentration of [U-^{14}C] uridine (2.5 μCi/ml). Transport of ra-

diolabeled uridine was terminated by addition of 1-ml portions of the ice-cold stop medium, the cells pelleted and then rapidly washed four times with 1-ml ice-cold portions of stop medium using a Fisher 235A microcentrifuge (10 sec, 12,000 × *g*). Incubation times of 30 sec were chosen such that the maximum intracellular concentrations of radioactivity did not exceed 15% of the extracellular levels in most instances (*see also* Results). For inhibition studies, nucleosides were added to cells at the same time as [U-^{14}C]uridine while NBMPR and dipyridamole were preincubated with cells for at least 10 min prior to the transport assay. Radioactivity associated with the cell pellet was determined as previously described (Jarvis & Martin, 1986). Transport rates were calculated after subtraction of ^{14}C-activity which became cell-associated due to nonmediated permeation. This correction was obtained by performing parallel experiments in the presence of 100 μM dipyridamole.

Kinetic constants of transport (apparent K_m and V_{max}) were determined by computer analysis of $[S]/V$ *versus* $[S]$ plots or by nonlinear least-squares fit of the equation,

$$V = V_{max} \cdot [S]/K_m + [S]$$

where V is the initial transport rate and $[S]$ is the nucleoside concentration (Barlow, 1983). Parameter values estimated by both methods were similar and are reported ±SE of the estimate. The correlation coefficients for the analyses were not less than 0.997.

Nitrobenzylthioinosine Binding

Membrane suspensions (50 μg of protein) were preincubated at 22°C in 3 ml 5 mM sodium phosphate buffer in the presence and absence of 5 μM NBTGR and when inhibitors of binding were evaluated, incubation mixtures also contained test compounds. After 10 min, 1-ml portions of medium containing [^{3}H]NBMPR (0.05 to 5.0 nM) were added. Incubations (30 min) were terminated by filtration (Whatman GF/C filters washed twice with 4 ml aliquots of ice-cold buffer; *see* Jarvis et al., 1983). [^{3}H]NBMPR binding to intact cells was determined by a centrifugation method as previously described (Jarvis & Young, 1980). Liquid scintillation counting was performed using an LKB/Wallac 1217 scintillation counter with automatic quench correction and disintegrations per minute conversion. Specific binding is defined as the difference in membrane or cell content of NBMPR in the presence and absence of 5 μM NBTGR.

Photoaffinity Labeling with [^{3}H]NBMPR

Photoaffinity labeling of rat erythrocyte membranes with [^{3}H]NBMPR (5 nM) was performed under equilibrium binding conditions in the presence of 25 mM dithiothreitol as previously described for human 'ghosts' (Wu et al., 1983*a*). Radioactivity associated with the membrane polypeptides was determined by SDS-polyacrylamide gel electrophoresis (9% acrylamide) by the method of Laemmli (1970) (*see also* Wu et al., 1983*a*).

Results

In preliminary time-course studies, it was demonstrated that initial rates of uridine influx by rat erythrocytes were measured by the cellular content of uridine after incubation intervals of 30 sec at 22°C (*see also* Jarvis et al., 1982*a*). Figure 1 compares the effect of the two nucleoside translocation inhibitors, NBMPR and dipyridamole, on uridine influx (100 μM) by rat erythrocytes. In contrast to human erythrocytes, where nucleoside transport is totally sensitive to NBMPR (Jarvis et al., 1982*b*), only about 35% of the transport activity in rat erythrocytes was inhibited by nanomolar NBMPR. The IC_{50} was 0.25 nM, transport inhibition reaching a plateau at 5 nM NBMPR. No further inhibition of uridine influx was observed until the concentration of NBMPR exceeded 1 μM. In a parallel experiment, NBMPR inhibition of uridine equilibrium exchange influx was also biphasic (*data not shown*). However, dipyridamole did not display a biphasic dose response curve for inhibition of uridine transport (IC_{50} value of 500 nM).

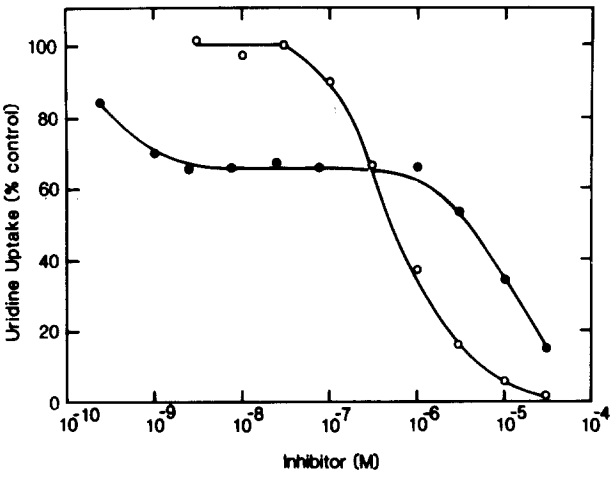

Fig. 1. Effect of NBMPR and dipyridamole on uridine influx by rat erythrocytes. Cells were preincubated with NBMPR (●) or dipyridamole (○) for 15 min before the addition of [^{14}C]uridine (final concentration of 0.1 mM). Results are given as a percentage of the control influx rate (1.13 mmol/liter cells per hr). Values are the average of triplicate estimates

The biphasic effect of NBMPR, as shown in Fig. 1, suggests that there may be two mechanisms for nucleoside transport in rat erythrocytes, one with a high sensitivity to inhibition by NBMPR and another with a low sensitivity to NBMPR inhibition. Measurements of nucleoside transport activity in the presence of 250 nM NBMPR were therefore used to define the component of transport insensitive to nanomolar concentrations of NBMPR. In attempts to define directly the NBMPR-sensitive component of transport, the sensitivity of uridine transport to *p*CMBS was examined. Table 1 demonstrates that low concentrations

Table 1. Effects of *p*-chloromercuriphenylsulfonate on uridine influx by rat erythrocytes in the presence and absence of nitrobenzylthioinosine.[a]

Incubation		Uridine influx (% control)
A	B	
*p*CMBS (1°C, 30 min)	NBMPR (22°C, 10 min)	
10 μM	—[b]	95
20 μM	—	76
50 μM	—	67
100 μM	—	50
200 μM	—	50
1000 μM	—	48
100 μM	0.25 nM	34
100 μM	1 nM	12
100 μM	2.5 nM	8
100 μM	7.5 nM	5

[a] Erythrocytes were subjected to consecutive incubations in the presence of *p*CMBS and NBMPR, after which the influx of [^{14}C]uridine (0.1 mM) was measured in triplicate as described in Materials and Methods. Cells were washed twice after incubation A to remove excess *p*CMBS. Results are given as a percentage of the control influx rate (1.02 mmol/liter cells per hr).
[b] — Denoted incubations with no additions.

Table 2. Reversal of *p*CMBS inhibition of uridine influx in rat erythrocytes by β-mercaptoethanol and dithiothreitol[a]

Incubation		Uridine influx (mmol/liter cells per hr)	
A (1°C, 15 min)	B (37°C, 30 min)	Control cells	NBMPR-treated cells
—[b]	—	0.48	0.27
*p*CMBS (50 μM)	—	0.22	0
*p*CMBS (50 μM)	DTT (1 mM)	0.28	0.11
*p*CMBS (50 μM)	DTT (5 mM)	0.40	0.22
*p*CMBS (50 μM)	β-mercaptoethanol (1 mM)	0.52	0.34
—	DTT (1 mM)	0.49	0.26
—	DTT (5 mM)	0.45	0.25
—	β-mercaptoethanol (1 mM)	0.56	0.36

[a] Erythrocytes (final hematocrit 3%) were subjected to two consecutive incubations (A and B) after which the influx of [^{14}C]uridine (0.1 mM) was measured in duplicate as described in Materials and Methods. Cells were washed twice after each incubation to remove excess reagent.
[b] — Denotes incubation with no addition.

of *p*CMBS (100 μM) resulted in 50% inhibition of the total uridine transport activity. Concentrations of *p*CMBS as high as 1.0 mM did not produce any further inhibition. Addition of low concentrations of NBMPR to cells pretreated with 100 μM *p*CMBS at 1°C resulted in inhibition of the residual transport flux with a dose response similar to that for NBMPR-sensitive transport in cells not pretreated with *p*CMBS (*see* Fig. 1). These data are consistent with the idea that the NBMPR-insensitive component of uridine transport in rat erythrocytes differs from the NBMPR-sensitive component in its sensitivity to *p*CMBS. Nucleoside transport activity following preincubation with 100 μM *p*CMBS was therefore used to define the component of transport sensitive to NBMPR.

Table 2 shows that incubation of *p*CMBS-treated erythrocytes at 37°C with dithiothreitol or β-mercaptoethanol resulted in recovery of the uridine transport activity, indicating that *p*CMBS was inhibiting NBMPR-insensitive transporter function by binding to membrane thiol groups. Control experiments confirmed that dithiothreitol and β-mercaptoethanol had little significant effect on uridine transport in untreated cells (Table 2).

The results presented in Fig. 2 demonstrate that uridine had the ability to protect erythrocytes against *p*CMBS inhibition in a dose-dependent manner (50% protection at approximately 0.4 mM uridine). Similar experiments with adenosine demonstrated that this nucleoside was able to protect the NBMPR-insensitive transport component against *p*CMBS inhibition of transport (75% protection at an extracellular adenosine concentration of 1.0 mM). In contrast, NBMPR had no effect on *p*CMBS inhibition. Control experiments using untreated cells showed that pretreatment of cells with uridine or adenosine, followed by washing at 37°C, had no effect on the influx of [^{14}C]uridine confirming that the nucleosides were protecting the transporter against *p*CMBS inhibition rather than causing an accelerative influx of [^{14}C]uridine. Further control studies demonstrated that maximum inhibition of uridine transport by 100 μM *p*CMBS was achieved by 1 min (71, 64, 62, 62 and 62% inhibition after 0.5, 1, 2, 5 and 15-min exposure to *p*CMBS).

The concentration dependence of uridine influx in the presence or absence of 100 μM dipyridamole for NBMPR- and *p*CMBS-treated rat erythrocytes compared to untreated cells is shown in Fig. 3. In the presence of dipyridamole, the concentration dependence of uridine influx was linear and similar for NBMPR- and *p*CMBS-treated cells (0.19 ± 0.04 and 0.26 ± 0.05 mmol/liter cells per hr at 1 mM, respectively, for cells from three animals). In the absence of dipyridamole, uridine transport by untreated, NBMPR- and *p*CMBS-treated cells was resolved into two components: a) a linear component and b) a saturable component which obeyed simple Michaelis-Menten kinetics. The kinetic constants for

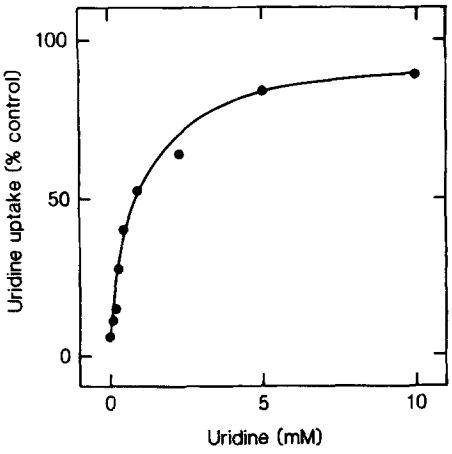

Fig. 2. Effect of uridine on *p*CMBS inhibition of uridine transport by the NBMPR-insensitive mechanism in rat erythrocytes. Cells were preincubated with 50 μM *p*CMBS at 1°C for 30 min in the presence and absence of varying concentrations of uridine, washed free of excess nucleoside and *p*CMBS at 37°C, and assayed for uridine uptake (final concentration 0.1 mM) at 22°C in the presence of 250 nM NBMPR. Values are the average of triplicate estimates and are expressed as percentages of the uridine transport rate measured in the absence of *p*CMBS. The control transport rate was 0.87 mmol/liter cells per hr at 22°C. The rate of uridine uptake (1 mM) at 1°C was 0.042 mmol/liter cells per hr

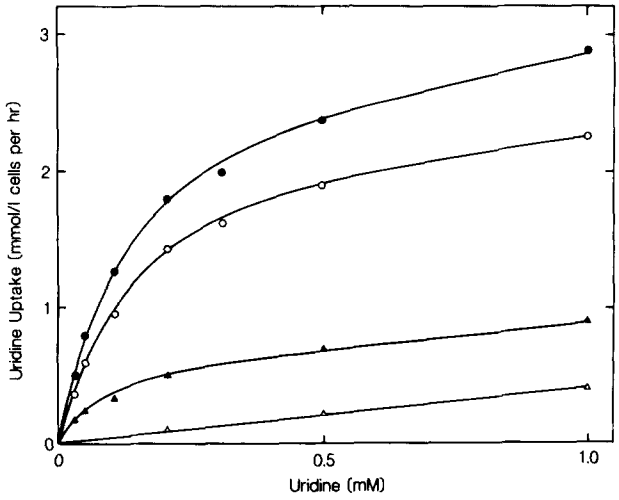

Fig. 3. Concentration dependence of uridine influx by control, NBMPR-treated and *p*CMBS-treated rat erythrocytes. [^{14}C]Uridine uptake by *p*CMBS-treated cells (▲) and untreated erythrocytes in the absence (●) or presence of either 100 μM dipyridamole (△) or 250 nM NBMPR (○) was determined as described in the text. Cells were preincubated with NBMPR and dipyridamole for 15 min before addition of radioactivity. Cells treated with *p*CMBS were incubated for 30 min at 1°C with 100 μM *p*CMBS and then washed twice to remove excess reagent

the saturable transport component were determined after subtraction of the linear component estimated in the presence of 100 μM dipyridamole. The apparent K_m values for uridine influx by untreated, NBMPR-treated and *p*CMBS-treated cells were 130 ± 10, 130 ± 10 and 72 ± 13 μM, respectively, with V_{max} values of 2.77 ± 0.05, 2.14 ± 0.04 and 0.56 ± 0.03 mmol/liter cells per hr. Calculation of the kinetic constants for the NBMPR-sensitive transport system by subtraction of the transport rates determined in the presence of NBMPR (250 nM) from the total transport rates yielded values similar to those estimated using *p*CMBS-treated cells (apparent K_m 73 ± 18 μM; V_{max} 0.51 ± 0.04 mmol/liter cells per hr). The mean values for the kinetic constants for NBMPR-sensitive (*p*CMBS-treated cells) and NBMPR-insensitive (NBMPR-treated cells) uridine transport from three separate experiments were 50 ± 18 (range 40 to 73) and 163 ± 28 (range 130 to 182) μM for the apparent K_m, respectively; with V_{max} estimates of 0.62 ± 0.09 and 2.27 ± 0.36 mmol/liter cells per hr, respectively (mean ± SEM). These results clearly demonstrate that there is a significant difference in the apparent affinity of the NBMPR-sensitive and -insensitive nucleoside transport mechanisms for uridine in rat erythrocytes.

To investigate the substrate specificity of the NBMPR-sensitive and -insensitive nucleoside transport mechanisms in rat erythrocytes, the effect of uridine, inosine and adenosine on the influx of radioactive uridine by NBMPR-treated and PCMBS-treated cells was examined in Fig. 4. In both cases, adenosine was the most effective inhibitor. Inosine and uridine appeared to be equally potent at inhibiting radioactive uridine influx by the NBMPR-sensitive route, while inosine was a more effective inhibitor than uridine for the NBMPR-insensitive mechanism. The apparent K_i values determined from the IC_{50} values for inhibition of uridine influx by the NBMPR-sensitive and NBMPR-insensitive transport mechanisms were, respectively, 62 and 164 μM for uridine, 53 and 48 μM for inosine and 12 and 27 μM for adenosine (mean values from two experiments). The apparent K_i values for uridine are identical to the apparent K_m values measured directly.

A complimentary approach to investigate the NBMPR-sensitive transport mechanism is to study the properties of high-affinity NBMPR binding to rat erythrocytes. Furthermore, since nucleoside transport by rat erythrocytes is only partially sensitive to NBMPR inhibition, it was considered possible that the characteristics of the rat erythrocyte NBMPR binding sites might be different from those in cells where NBMPR totally inhibits transport activity. [^{3}H]NBMPR binding to rat erythrocyte ghosts was resolved into two components: a) a saturable association responsible for the binding of 0.65

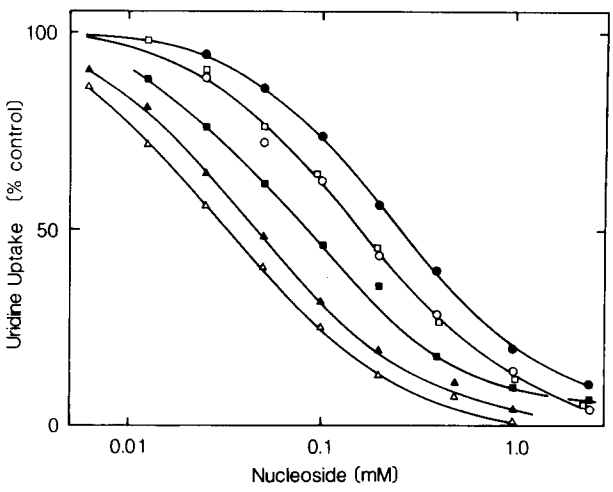

Fig. 4. Effect of nucleosides on NBMPR-sensitive and NBMPR-insensitive uridine uptake by rat erythrocytes. To determine the NBMPR-sensitive (*p*CMBS resistant) component of transport, rat erythrocytes were incubated in the presence (open symbols) of 100 μM *p*CMBS for 30 min at 1°C and washed twice to remove excess reagent. NBMPR-insensitive uridine transport (closed symbols) was measured by preincubating cells with 250 nM NBMPR. [^{14}C]Uridine (final concentration 0.1 mM) and unlabeled nucleosides (●, ○, uridine; ■, □, inosine; and ▲, △, adenosine) were added simultaneously and uridine uptake was determined as described in the text. Control values for uridine influx by NBMPR-treated and *p*CMBS-treated cells were 0.58 and 0.69 mmol/liter cells per hr, respectively. Nonsaturable uptake, determined in the presence of 100 μM dipyridamole, was subtracted from the uptake values and represented 3% of the total flux for the controls

± 0.25 (5) pmol/mg protein with an apparent K_d of 0.046 ± 0.025 (5) nM and b) a nonsaturable component responsible for the binding of 0.051 ± 0.016 (5) pmol/mg protein at 1 nM (means ± SE (*n*)). The saturable component of binding was abolished by pretreating membranes with NBTGR (5 μM), and the Hill coefficients calculated for the saturable binding data were not significantly different from unity, indicating that NBMPR was binding to a single class of sites. Mean high-affinity NBMPR binding activities for four intact erythrocyte samples were 6.2 ± 1.0 pmol/ml cells with a corresponding V_{max} value for NBMPR-sensitive uridine zero-trans influx of 158 ± 16 pmol/mol cells per sec. If each NBMPR binding site is assumed to represent a single NBMPR-sensitive nucleoside transport site, these values give estimated turnover numbers for uridine influx of 25 ± 6 molecules/site per sec at 22°C.

The effect of varying concentrations of nucleosides and nucleoside transport inhibitors on site-specific binding of NBMPR to rat erythrocyte membranes was further explored in Fig. 5. Adenosine was a more effective inhibitor than thymidine and uridine, IC_{50} values of 0.067, 0.40 and 0.65 mM for adenosine, thymidine and uridine, respectively. This result is consistent with the relative affinities of these nucleosides for the NBMPR-sensitive transport mechanism (*see* Fig. 4). In other experiments, uridine was shown to competitively inhibit

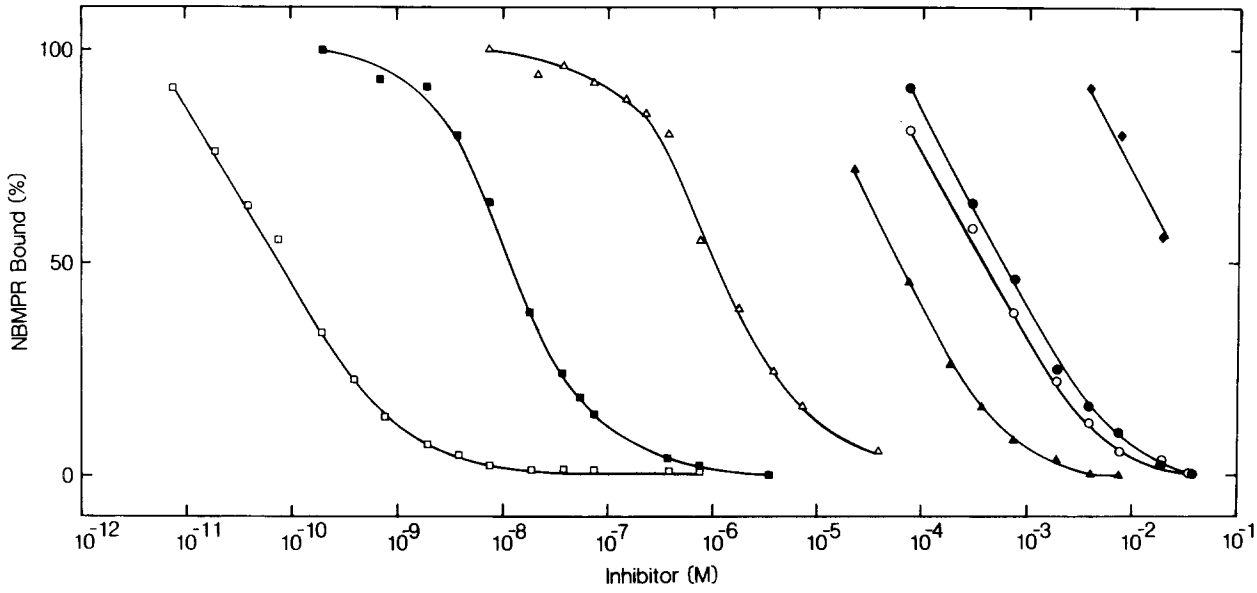

Fig. 5. Effects of nucleosides and nucleoside transport inhibitors on [^{3}H]NBMPR binding to rat erythrocyte membranes. Site-specific (NBTGR-sensitive) [^{3}H]NBMPR binding (0.1 nM) to rat erythrocyte membranes was measured in the presence of NBTGR (□), dilazep (■), dipyridamole (△), adenosine (▲), thymidine (○), uridine (●) and thymine (◆). Results are plotted as a percentage of control high-affinity NBMPR activity in the absence of inhibitors (0.33 pmol/mg protein). Values are means of triplicate estimates

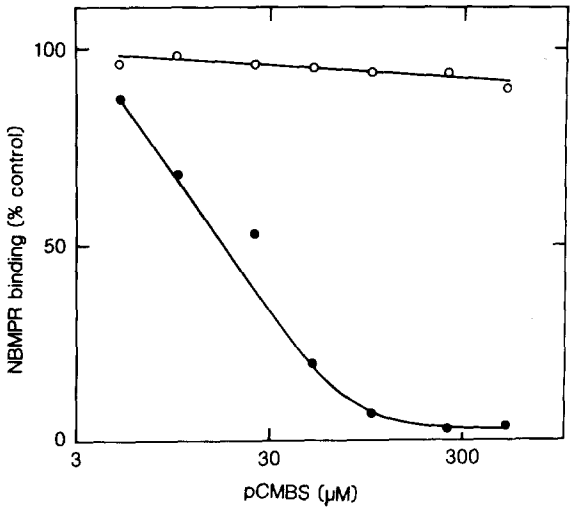

Fig. 6. *p*CMBS inhibition of [^{3}H]NBMPR binding to intact rat erythrocytes and unsealed erythrocyte ghosts. Samples were pretreated with varying concentrations of *p*CMBS at 1°C for 30 min, washed free of excess reagent and assayed for high-affinity (NBTGR-sensitive) NBMPR binding activity (0.1 nM) as described in Materials and Methods. Values are the means of triplicate estimates and are expressed as percentages of control NBMPR binding activities measured in the absence of organomercurial. Symbols: ○, intact cells; ●, ghosts

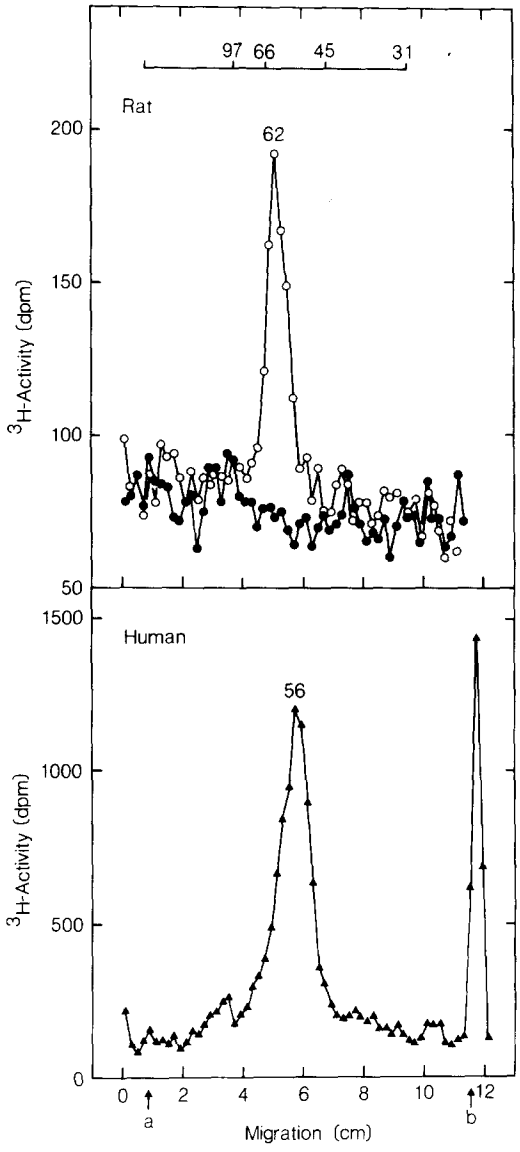

Fig. 7. Photoaffinity labeling of rat and human erythrocyte membranes with [^{3}H]NBMPR. Rat erythrocyte membranes equilibrated with 5 nM [^{3}H]NBMPR in the presence (●) or absence (○) of 5 μM NBTGR and human ghosts (▲) equilibrated with 50 nM [^{3}H]NBMPR were supplemented with 25 mM dithiothreitol and exposed to ultraviolet light at 4°C for 20 sec. Samples were subjected to SDS-polyacrylamide gel electrophoresis as described in the text. Positions of the molecular weight standards are from the same slab gel. *a*, Stacking gel-running gel interface; *b*, tracking-dye

[^{3}H]NBMPR binding to rat membranes with an inhibition constant of 0.50 mM (*data not shown*). The nucleobase, thymine, had little effect on site specific binding (IC_{50} > 20 mM). In contrast to human erythrocytes where the potency for inhibition of NBMPR binding by NBTGR, dilazep and dipyridamole differ by less than 10-fold (Hammond & Clanachan, 1984), the IC_{50} values for inhibition for binding to rat erythrocyte membranes differed by 4 orders of magnitude (0.08, 12 and 1000 nM for NBTGR, dilazep and dipyridamole, respectively). The lower potency of dilazep and dipyridamole for inhibition of [^{3}H]NBMPR binding has also been observed for cortical, liver, cardiac muscle and lung rat membranes when compared to the membranes prepared from guinea pig tissues (Hammond & Clanachan, 1984; Shi et al., 1984; Wu & Young, 1984; Williams et al., 1984; Verma & Marangos, 1985).

The NBMPR-sensitive nucleoside transporter in human and fetal sheep erythrocytes has been demonstrated to be chemically asymmetric with respect to *p*CMBS inhibition of transport activity, inhibition only occurring when the organomercurial has access to the cytoplasmic membrane surface (Jarvis & Young, 1982; Tse et al., 1985). To investigate whether the NBMPR-sensitive nucleoside transporter in rat erythrocytes exhibited similar chemical asymmetry, the effect of *p*CMBS on high-affinity NBMPR binding to unsealed erythrocyte ghosts and intact cells was compared in Fig. 6. *p*CMBS was an effective inhibitor of high-affinity NBMPR binding activity in 'ghosts' (50% inhibition at approximately 20 μM), but did not inhibit NBMPR binding to intact cells. In another experiment, the kinetic constants for NBMPR binding were similar for control and 100 μM *p*CMBS-treated

erythrocytes (apparent K_d 34 ± 2 and 36 ± 7 pM; B_{max} 5.72 ± 0.08 and 5.82 ± 0.27 pmol/mol cells for control and *p*CMBS-treated cells). The inability of *p*CMBS to inhibit NBMPR binding by intact cells is consistent with the lack of effect of *p*CMBS on uridine transport mediated by the NBMPR-sensitive transporter in intact erythrocytes (*see* earlier).

Previous studies (Wu et al., 1983*a*,*b*; Young et al., 1983) have described the use of NBMPR as a covalent photoaffinity probe of the nucleoside transporter in human erythrocytes. Figure 7 compares the photoincorporation of [^{3}H]NBMPR into membranes prepared from human and rat erythrocytes. In agreement with previous results (Wu et al., 1983*a*,*b*), the major peak of radiolabeling in human erythrocyte membranes migrated in the band 4.5 region of the polyacrylamide gel with a peak apparent molecular weight of 56,000. Similarly, for rat erythrocytes a single symmetrical peak of radiolabeling in the band 4.5 region of the electropherogram was observed. However, the rat erythrocyte peak was sharper than the human erythrocyte peak and had a significantly higher apparent molecular weight (M_r 62,000). Covalent incorporation of [^{3}H]NBMPR into these polypeptides was abolished when photolysis was carried out in the presence of NBTGR. Both preparations exhibited nonspecific labeling in the lipid region of the gel. In other experiments, the effect of endoglycosidase F, an enzyme which cleaves glycans of both the high-mannose and complex type, on the apparent molecular weight of the [^{3}H]NBMPR photolabeled proteins in rat erythrocytes was compared to that in human red cells. In both cases endoglycosidase F digestion caused a sharpening of the ^{3}H-peak and a shift to a lower apparent M_r (M_r 47,000 for both human and rat erythrocytes) (*data not shown*).

Discussion

The results presented in this paper suggest that rat erythrocytes have two distinct components for neucloside transport which can be distinguished on the basis of their sensitivity to NBMPR and the slowly permeating organomercurial *p*CMBS. These findings extend the previous observations with cultured mammalian cells which have demonstrated multiple forms of nucleoside transport (Eilam & Cabantchik, 1977; Bibi et al., 1978; Heichal et al., 1978; Belt, 1983; Plagemann & Wohlhueter, 1984*a*).

The first transport component, which we define as NBMPR-sensitive, was inhibited by nanomolar concentrations of NBMPR (IC_{50} 0.25 nM which gives an estimated K_i value of 83 pM) and represented approximately 20% of the total uridine flux at a saturating concentration of nucleoside. Inhibition by NBMPR was associated with high-affinity NBMPR binding to the erythrocyte membrane which was blocked by nucleoside transport inhibitors and nucleosides. Covalent incorporation of [^{3}H]NBMPR, following exposure to UV light, resulted in a single symmetrical peak of radiolabeling in the band 4.5 region of SDS-electropherograms which was abolished when photolysis was carried out in the presence of NBTGR. The apparent M_r of the rat erythrocyte radioactive peak was significantly higher than that of the human erythrocyte (62,000 compared to 56,000; Fig. 7). Endoglycosidase F digestion of [^{3}H]NBMPR-labeled proteins caused a shift in mobility of the ^{3}H-peak to an apparent M_r for 47,000 for both human and rat demonstrating that the M_r discrepancy is primarily due to glycosylation differences. *p*CMBS had no effect on NBMPR-sensitive uridine transport and NBMPR binding to intact cells but totally blocked NBMPR binding to disrupted membranes where both sides of the cell membrane are accessible to *p*CMBS. This result suggests that the NBMPR-sensitive system of nucleoside transport has a *p*CMBS-sensitive thiol group located on the inner surface of the cell membrane. These combined properties of the NBMPR-sensitive component of nucleoside translocation in rat erythrocytes are similar to the nucleoside transporter in human and nucleoside-permeable sheep erythrocytes, cell types in which nucleoside translocation is totally sensitive to NBMPR (Jarvis & Young, 1982; Young & Jarvis, 1983; Tse et al., 1985). One notable difference is that the turnover number for the NBMPR-sensitive nucleoside transport system in rat erythrocytes is 25 ± 6 molecules/site per sec at 22°C, a value similar to that recently reported by Plagemann and Wohlhueter (1985) but fivefold less than that of other mammalian erythrocyte NBMPR-sensitive nucleoside transporters (Jarvis & Young, 1982; Jarvis et al., 1982*a*). It was previously reported (Jarvis et al., 1982*a*) that the turnover number for the rat and human erythrocyte nucleoside transport systems were similar, but this earlier report failed to investigate the detailed sensitivity of rat erythrocyte nucleoside transport to NBMPR.

The second nucleoside transport component in rat erythrocytes, which we define as NBMPR-insensitive, was not inhibited by concentrations of NBMPR as high as 1 μM. In contrast to the NBMPR-sensitive system, low concentrations of *p*CMBS (25 to 1000 μM) at 1°C were able to inhibit transport under conditions where entry of *p*CMBS into the cell is minimal (Rothstein, 1970). Inhibition by *p*CMBS was reversed by dithiothreitol and β-mercaptoethanol and the nucleoside uridine and

adenosine gave considerable protection against inhibition. Since the V_{max} for uridine transport into rat erythrocytes at 1°C is extremely low (less than 0.045 mmol/liter cells per hr), the protective effect of nucleosides against *p*CMBS inhibition occurred under conditions where there was little transport of nucleoside into the cell. It is therefore likely that the *p*CMBS-sensitive thiol groups are located on the exterior surface of the cell membrane within the nucleoside binding site of the transport system, a suggestion supported by the finding that maximum inhibition of uridine transport by *p*CMBS is obtained within 1 min. For cultured mammalian cells, the NBMPR-sensitive and -insensitive nucleoside transport mechanisms have been variously reported to exhibit both differences in sensitivity to organomercurials (Belt, 1983; Belt & Noel, 1985) and to exhibit no differences (Plagemann & Wohlhueter, 1984*a*,*b*). Much of this confusion is probably related to the high temperatures (20 to 37°C) used during the incubation with *p*CMBS and *p*-hydroxymercuribenzoate in these cultured cell studies. Indeed, recent studies have shown that the difference in sensitivities for *p*CMBS inhibition of nucleoside transport between Walker 256 cells (NBMPR-insensitive) and S49 cells (NBMPR-sensitive) are not apparent when incubations are performed at 37°C (Belt & Noel, 1985).

The two transport mechanisms in rat erythrocytes also differed in their substrate affinities. Although both transport systems had a broad specificity, the affinity of the NBMPR-insensitive system was approximately threefold less for uridine and adenosine than the NBMPR-sensitive system (Figs. 3 and 4). The affinity for inosine, as determined from the inhibition of uridine transport, was similar for both transport mechanisms. These results are not in agreement with reports using cultured mammalian cells which suggested that the substrate affinities for the two types of transport, with the exception of CHO cells, were indistinguishable (Belt, 1983; Plagemann & Wohlhueter, 1984*a*). However, these workers compared the kinetic constants for total uridine influx (fitted to a single component) with those measured in the presence of NBMPR. In contrast, both nucleoside transporters in rat erythrocytes appeared to be equally sensitive by dipyridamole inhibition, as judged from the monophasic inhibition curve presented in Fig. 1.

In conclusion, the present results suggest that there are two facilitated diffusion mechanisms for nucleoside transport in rat erythrocytes: one sensitive to nanomolar concentrations of NBMPR but insensitive to *p*CMBS on the outer surface of the cell, and the other insensitive to NBMPR but sensitive to externally located *p*CMBS. Cabantchik and colleagues have also suggested the presence of two parallel nucleoside transport systems in cultured hamster cells with different susceptibilities to NBMPR and thiol reactive reagents (Bibi et al., 1978; Heichal et al., 1978). The substrate affinities of these two systems in rat erythrocytes also differ. Functionally, the two transporters are sufficiently similar to suggest that they represent the products of closely related genes. Alternatively, they may reflect two conformations of a single protein, one which binds NBMPR and one which cannot bind NBMPR.

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