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NUCLEOSIDE TRANSPORT IN CULTURED MAMMALIAN CELLS

MULTIPLE FORMS WITH DIFFERENT SENSITIVITY TO INHIBITION BY NITROBENZYLTHIOINOSINE OR HYPOXANTHINE

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The zero-*trans* influx of 500 μM uridine by CHO, P388, L1210 and L929 cells was inhibited by nitrobenzylthioinosine (NBTI) in a biphasic manner; 60–70% of total uridine influx by CHO cells and about 90% of that in P388, L1210 and L929 cells was inhibited by nmolar concentrations of NBTI (ID_{50} = 3–10 nM) and is designated NBTI-sensitive transport. The residual transport activity, designated NBTI-resistant transport, was inhibited by NBTI only at concentrations above 1 μM (ID_{50} = 10–50 μM). S49 cells exhibited only NBTI-sensitive uridine transport, whereas Novikoff cells exhibited only NBTI-resistant uridine transport. In all instances NBTI-sensitive transport correlated with the presence of between $7 \cdot 10^4$ and $7 \cdot 10^5$ high-affinity NBTI binding sites/cell (K_d = 0.3–1 nM). Novikoff cells lacked such sites. The two types of nucleoside transport, NBTI-resistant and NBTI-sensitive, were indistinguishable in substrate affinity, temperature dependence, substrate specificity, inhibition by structurally unrelated substances, such as dipyridamole or papaverine, and inhibition by sulfhydryl reagents or hypoxanthine. We suggest, therefore, that a single nucleoside transporter can exist in an NBTI-sensitive and an NBTI-resistant form depending on its disposition in the plasma membrane. The sensitive form expresses a high-affinity NBTI binding site(s) which is probably made up of the substrate binding site plus a hydrophobic region which interacts with the lipophilic nitrobenzyl group of NBTI. The latter site seems to be unavailable in NBTI-resistant transporters. The proportion of NBTI-resistant and sensitive uridine transport was constant during proportion of NBTI-resistant and sensitive uridine transport was constant during progression of P388 cells through the cell cycle and independent of the growth stage of the cells in culture. There were additional differences in uridine transport between cell lines which, however, did not correlate with NBTI sensitivity and might be related to the species origin of the cells. Uridine transport in Novikoff cells was more sensitive to inhibition by dipyridamole and papaverine than that in all other cell lines tested, whereas uridine transport in CHO cells was the most sensitive to inactivation by sulfhydryl reagents.

Introduction

On the bases of uniform kinetic properties and substrate specificity and the isolation of a single-step, transport-deficient mutant of mouse T-cell lymphoma S49 cells, mammalian cells have been postulated to possess a single nucleoside transporter that transports all natural ribo- and deoxyribonucleosides, albeit with different efficiencies (for review, see Ref. 1). The Michaelis-Menten constants observed with various types of cultured

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cells, regardless of species or tissue of origin, are lowest for purine nucleoside transport (100–200 μM), intermediate for uridine and thymidine transport (150–300 μM) and above 500 μM for cytidine and deoxycytidine transport [1]. Nucleoside transport by many types of cells has been found to be exquisitely sensitive to inhibition by 6([4-nitrobenzyl]thio)-9-β-D-ribofuranosylpurine (nitrobenzylthioinosine, NBTI) (K_i about 1 nM; Refs. 2–7). The potent inhibition of nucleoside transport coincides with the presence of high-affinity binding sites on cells. For example, uridine transport capacity of erythrocytes from various species correlates with the number of high-affinity NBTI binding sites on the cells (ranging from about 20 sites/cell for adult sheep and guinea pig red cells to $1 \cdot 10^4$ sites/cell for human and rabbit red cells [8]) and a similar correlation is observed for the 100-fold difference in uridine transport capacity between fetal and adult sheep erythrocytes [9]. Furthermore, a mutant of S49 cells deficient in nucleoside transport lacks high-affinity NBTI binding sites [10], whereas wild-type S49 cells and cells of a number of other cell lines possess between $1 \cdot 10^5$ and $5 \cdot 10^5$ NBTI binding sites/cell [4,7,10,11]. Binding equilibria with [^{3}H]NBTI support the idea of direct competition with transport substrates: despite a 10^5-fold difference in affinity, [^{3}H]NBTI is displaced from its binding site by high concentrations of nucleosides. A general view, therefore, has arisen that high-affinity NBTI binding measures functional nucleoside transporters in mammalian cells [9].

There are, however, some conspicuous exceptions to this generality. Nucleoside uptake by HeLa and MTC hamster cells has been found to be inhibited by NBTI to maximally 50 to 90% with variations depending on the cell line [4,12]. This partial inhibition of uptake presumably reflects partial inhibition of transport, although these studies did not distinguish effects on transport and phosphorylation. Using cells unable to phosphorylate thymidine, we have found that transport per se was inhibited by 100 nM NBTI to about 90% in Chinese hamster ovary (CHO) and mouse L and P388 cells, to about 77% in HeLa cells and not at all in Novikoff rat hepatoma cells [2]. Belt recently reported [13,14] that uridine influx in S49 and RPMI 6410 cells was inhibited > 98% by 1 μM NBTI, but only about 90% and 80% in P388 and L1210 cells, respectively, whereas in Walker 256 rat carcinoma cells, like in Novikoff cells, it was not affected. Furthermore, her data indicated that NBTI-resistant uridine influx in L1210 cells did not simply reflect non-mediated permeation, but was mediated by a NBTI-resistant, broadly specific, *p*-hydroxymercuribenzenesulfonate-sensitive transporter, just like that of Novikoff cells [1].

The question, therefore, arises of whether mammalian cells really possess only a single transporter, or whether there may exist two structurally distinct transporters, one sensitive to NBTI and one resistant. A middle hypothesis would be that there exists only one transporter which manifests two conformational forms, only one of which is capable of binding NBTI with high affinity, and, hence, very sensitive to inhibition.

We have addressed these questions by comparing various cell lines with respect to binding of radiolabeled NBTI, the presence of NBTI inhibitable and resistant uridine transport, and the sensitivity of this transport to inhibition by other nucleosides and hypoxanthine, as well as by structurally unrelated inhibitors of nucleoside transport including sulfhydryl reactive agents.

Experimental procedures

Materials. [5-^{3}H]Uridine and [G-^{3}H]NBTI were obtained from Moravek Biochemicals (Brea, CA, U.S.A.) and diluted to desired specific radioactivities with unlabeled uridine or NBTI. Unlabeled nucleosides and *p*-hydroxymercuribenzoate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and unlabeled NBTI from Calbiochem (San Diego, CA, U.S.A.). Dipyridamole (Persantin) was a gift from Geigy Pharmaceuticals (Yonkers, NY, U.S.A.).

Cell culture. Wild-type Novikoff rat hepatoma cells and uridine kinase-deficient (1-14-7; Ref. 15) and hypoxanthine phosphoribosyltransferase-deficient (1-22; Ref. 16) variants thereof were propagated in suspension culture in Swim's medium 67 as described previously [17]. Wild-type Chinese hamster ovary (CHO) cells, CHO-DAP12 cells, CHO-AR7 cells, CHO-Tub^r cells, mouse L929-2 cells, and mouse leukemia P388 and L1210 and

lymphoma S49 cells were propagated in a similar manner in Eagle's minimum essential medium for suspension culture supplemented with serum, D-glucose and non-essential amino acids as described previously [17]. CHO-DAP12 cells are deficient in adenine and hypoxanthine phosphoribosyltransferases, CHO-AR7 and CHO-Tub^r in adenosine kinase [18] and L929-2 is a variant of mouse NCTC929 cells which is deficient in adenine phosphoribosyltransferase [19]. All cell lines were demonstrated to be free of mycoplasma contamination by uridine/uracil incorporation [20] and cultural methods. For experiments, cells were harvested by centrifugation from mid to late exponential phase cultures and suspended to $8 \cdot 10^6$ to $2 \cdot 10^7$ cells/ml of basal medium 42B (BM42B).

Nucleoside transport. Nucleoside transport was measured in cell suspensions at 25°C as we have described previously [1,21]. Time-courses (comprising 15 points) of transmembrane equilibration of radiolabeled uridine were determined under zero-*trans* or equilibrium exchange conditions by rapid kinetic techniques. Data were evaluated by fitting integrated rate equations, based on a simple carrier with directional symmetry and equal mobility of empty and nucleoside-loaded carrier [1,22], as appropriate for zero-*trans* or isotopic exchange. In experiments to determine Michaelis-Menten parameters, six to eight substrate concentrations were employed and the parameters extracted by least-squares regression. In other experiments, where only initial velocities at a single permeant concentration (usually 320 or 500 μM) were of interest, an integrated rate for zero-*trans* influx was fitted with K_m fixed at 250 μM, and the slope at $t = 0$ taken as initial velocity (v_{12}^{zt}).

In experiments to test the effects of various substances on nucleoside transport cell suspensions were treated as follows: sulfhydryl reagents were preincubated with cells for 20 min at 37°C before transport was measured at 25°C; NBTI, dipyridamole and papaverine were added to cell suspensions (at 25°C) at least 2 min prior to transport assay; unlabeled nucleosides or hypoxanthine were added simultaneously with the labeled transport substrate. In all cases, flux is expressed as pmol of labeled permeant per second and μl of cellular H_2O [21].

Equilibrium binding of NBTI. Our method of measuring equilibrium binding has also been described in detail previously [7]. Samples of cell suspensions were mixed with 12 concentrations (0.15 to 500 nM) of [^{3}H]NBTI (final density between $3 \cdot 10^6$ and $2 \cdot 10^7$ cells/ml of BM42B) and, after about 40 min at 25°C, triplicate samples were taken from the suspension (= bound + free NBTI) and a centrifugally cleared supernate (= free NBTI) for scintillation counting. Measured radioactivity was converted to concentrations of free NBTI and bound NBTI, and the following equation was fitted to the pooled data:

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

where L_b = concentration of bound ligand, L_f = concentration of free ligand, N = number of binding sites per liter, K_d = dissociation constant and k' = a coefficient of non-saturable binding. The equation corresponds to a single, saturable binding site plus a non-specific binding component [7].

Data analysis. The theoretical equations were fitted to data by a generalized least-squares regression program based on the algorithm of Dietrich and Rothmann [23] and implemented on a Hewlett-Packard 9825 computer. Parameter values are reported ± S.E. of the estimate.

Results

Fig. 1 shows representative time courses of uptake of 320 μM [^{3}H]uridine by P388 cells in the presence of the indicated concentrations of dipyridamole or NBTI and illustrates the general method of measuring zero-*trans* influx in all experiments presented. Chromatographic analysis of the acid soluble pools of the labeled cells showed that after 1 min of incubation less than 10% of the intracellular radioactivity was associated with nucleotides (data not shown). Thus the uptake time courses reflected transmembrane equilibration of unmodified uridine (see also Ref. 1). In these and similar experiments with other cell lines and/or inhibitors initial zero-*trans* entry velocities (v_{12}^{zt}) were estimated as the zero-time slopes of the time-courses of transmembrane equilibration [1].

In Fig. 2 the v_{12}^{zt} values for uridine transport in a number of cell lines are plotted as the function

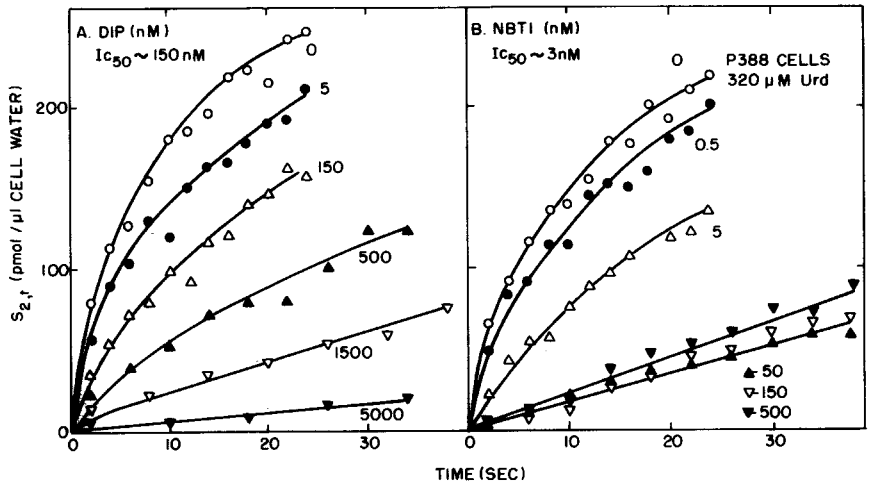

Fig. 1. Effect of dipyridamole (DIP) and NBTI on the zero-*trans* influx of uridine in P388 cells. Samples of a suspension of $1.5 \cdot 10^7$ P388 cells/ml of BM42B were supplemented with the indicated concentrations of dipyridamole (A) or NBTI (B). Then the time-course of transmembrane equilibration of 320 μM [^{3}H]uridine (Urd) (1.7 cpm/pmol) was measured at 25°C and the initial velocities of zero-*trans* entry (v_{12}^{zt}) were computed by integrated rate analysis of the data as described under Experimental procedures. For the control cells, $v_{12}^{zt} = 30$ pmol/μl cell water per s. The v_{12}^{zt} values for the inhibitor-treated cells are summarized in Fig. 2A and B as percent of control. $S_{2,t}$, intracellular substrate concentration at time t.

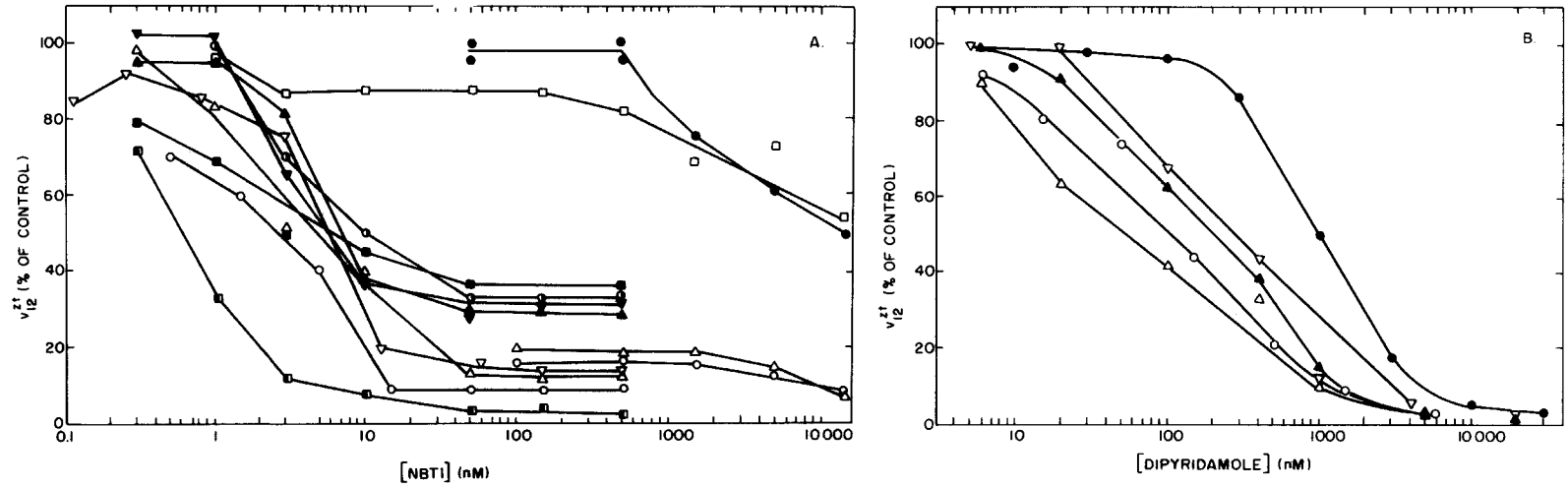

Fig. 2. Zero-*trans* uridine influx in various cell lines as a function of concentration of NBTI (A) and dipyridamole (B). The values for P388 cells in A and B were calculated from the data in Fig. 1. All other experiments were conducted in the same manner as illustrated in Fig. 1. The v_{12}^{zt} values for untreated control cells ranged from 17 to 44 pmol/μl cell water per s in the various experiments. ●———●, N1S1-67 wild type; □———□, N1S1-67 (1-14-7); ▲———▲, CHO wild type; ▼———▼, CHO-DAP-12; ■———■, CHO-AR7; ◑———◑, CHO-Tub^r; ○———○, P388; △———△, L929-2; ▽———▽, L1210; ◧———◧, S49.

of the concentration of inhibitor. The uridine concentrations employed, 320 or 500 μM, were sufficient to achieve about 56 or 66% of maximal influx, respectively. In all instances the inhibition of uridine influx was dependent on inhibitor concentration, and most cell lines investigated exhibited similar sensitivities to NBTI (Fig. 2A, IC_{50} = 3–10 nM), dipyridamole (Fig. 2B, IC_{50} = 50–300 nM) and papaverine (IC_{50} = 2–4 μM, data not shown). One exception was wild-type Novikoff rat hepatoma cells. Urd influx in these cells was significantly inhibited by NBTI only at concentrations above 0.5 μM; the IC_{50} was about 1000-fold higher than that for all other cell lines investigated. Uridine influx in wild-type Novikoff cells was also more resistant to inhibition by dipyridamole (Fig. 2B, IC_{50} = 1 μM) and papaverine (IC_{50} = 20 μM, data not shown) than that of the other cell lines, but the differences in sensitivity were much smaller than observed for NBTI. There was also a noteworthy difference between the pattern of inhibition of uridine influx by NBTI on the one hand, and dipyridamole and papaverine on the other hand. The degree of inhibition by dipyridamole and papaverine increased progressively with increase in concentration until influx was almost completely inhibited. In the case of NBTI, such pattern of inhibition was only observed for mouse lymphoma S49 cells which were also somewhat more sensitive to inhibition by NBTI than the other cell lines (Fig. 2A). The inhibition of Urd transport by NBTI in these other NBTI-sensitive lines, on the other hand, was biphasic. Inhibition leveled off above 10–50 nM reaching a level of inhibition between 60 and 92% depending on the cell line. The residual transport activity by P388 and L1210 cells, however, became inhibited at concentrations of NBTI above 1 μM, in a pattern similar to that observed for Novikoff cells (Fig. 2A). The dose-response curve for L1210 is similar to that reported by Belt [14]. A similar biphasic pattern of inhibition of uridine uptake by NBTI has also been reported for HeLa cells [6]. The dose-response patterns for NBTI inhibition of uridine transport for individual NBTI-sensitive cell lines varied somewhat between experiments. This applied to both the progressive increase in inhibition below 10 nM NBTI and the level of inhibition attained between 20 and 500 nM NBTI. The latter, however, fell consistently between 80 and 92% inhibition for P388, L1210 and L929-2 cells and between 60 and 75% for wild-type CHO cells and three independently derived variants of these cells (AR-7, DAP-12, and Tub^r) exhibited a similar pattern of NBTI inhibition as wild-type cells (Fig. 2A). In addition, the level of inhibition of uridine influx by 50 and 500 NBTI was identical in seven independent clones of wild-type CHO cells (60–70%) and the pattern of inhibition in CHO, L929-2 and P388 cells was constant during at least one year of continuous propagation in cell culture.

The data of Fig. 2A show slight (approx. 12%) inhibition or Urd influx in the uridine kinase-deficient Novikoff cells (1-14-7) at NBTI concentrations in the range of 50 to 500 nM. This component of inhibition has not been apparent in wild-type Novikoff cells, but we have observed it consistently in several experiments with 1-14-7 cells. At concentrations above 500 nM NBTI, the pattern of inhibition in 1-14-7 cells was similar to that in wild-type cells.

Additional results lead us to believe that the unexpected difference between 1-14-7 cells and their parental type is not a consequence of contamination of the 1-14-7 cells with another cell type with NBTI-inhibitable nucleoside transport. First, we have repeated the transport measurements after the cells were propagated for 10 days in the presence of 10 μM 5-fluorouridine. Since we do not carry any 5-fluorouridine-resistant variants other than the 1-14-7 Novikoff cells, this treatment should have eliminated any wild-type cells as well as all other potential contaminating cells. Second, no contaminating cells were detected by karyotype analysis; the karyotype of 1-14-7 cells was indistinguishable from that previously reported for wild-type Novikoff cells [24] (average 39 chromosomes/cell). Furthermore, v_{12}^{zt} for 500 μM uridine was similar for wild-type and 1-14-7 Novikoff cells (25–35 pmol/μl cell water per s), supporting our contention that uridine uptake at high concentrations is a valid measure of transport, irrespective of the cells' ability to phosphorylate uridine. Thus, the absence in wild-type cells of inhibition at lower NBTI concentrations would seem not to arise from metabolic interference with transport measurements in cells capable of uridine phosphorylation.

The variation in NBTI-inhibition patterns among cell lines and among experiments with a single cell line suggested that the proportion of NBTI-sensitive nucleoside transporter is subject to modulation in various physiological conditions. We therefore harvested P388 and CHO cells at different densities, corresponding to different stages of culture growth, but found no difference in NBTI sensitivity from asynchronous cells in early, mid or late exponential phase or early stationary phase (Table I).

Nor did position in the cell cycle of a synchronous culture of P388 cells influence the proportion of NBTI-inhibitable transport. Fig. 3 shows the results of assays of uridine transport in P388 cells at various times after release from a double hydroxyurea block [24]. Zero-*trans* influx was 90% inhibited by 50 and 500 μM NBTI regardless of the cell cycle stage (early S, late S to G_2, M and G_1).

The results in Fig. 2A demonstrated the presence of at least two types of nucleoside transport in various cell lines, one highly sensitive to inhibition by NBTI (IC_{50} = 3–10 nM) and the other one relatively resistant (IC_{50} = 10–20 μM). Practically all uridine transport in Novikoff and Walker 256 [14] cells and 85–90% of the total in the uridine kinase-deficient Novikoff variant is NBTI-resistant, whereas only between 10 to 20% of the total in P388, L1210 and L929-2 cells and 30–40% in CHO cells is resistant and no NBTI-resistant uridine transport is detectable in S49 cells. The data in Fig. 4 and Table II show that in all cases high sensitivity to inhibition by NBTI correlated with the presence of high-affinity binding sites for NBTI. NBTI binding has been previously analyzed in HeLa and CHO cells and found to be composed of a saturable high-affinity component (K_d = 0.1–1 nM) and a non-saturable component [4,7]. Our data extend these findings to P388, L1210, L929-2 and S49 cells. Because of the wide range of NBTI concentrations that needed to be analyzed, the binding data are presented in the form of a log-log plot of bound NBTI versus free NBTI (Fig. 4) to allow direct comparison between saturable and non-specific binding and of NBTI binding in the various cell lines. The number of high-affinity binding sites varied from about $0.7 \cdot 10^5$/S49 cell to about $7 \cdot 10^5$/L929-2 cell and correlated roughly with the size of the cells (Table II). Although an exact analysis is not possible on the basis of these data, they suggest that the density of binding sites per surface area is similar in the various cell lines, as is also suggested by the similar transport capac-

TABLE I

NBTI-SENSITIVE AND NBTI-RESISTANT URIDINE TRANSPORT IN CHO AND P388 CELLS AS FUNCTION OF GROWTH STAGE

Cultures were initiated at about $1 \cdot 10^5$ cells/ml. At the indicated cell densities (equivalent to early, mid and late exponential phase and early stationary phase), an approximately equal number of cells was collected by centrifugation, suspended to about $1 \cdot 10^7$ wild-type CHO cells or $1.3 \cdot 10^7$ P388 cells/ml of BM42B and assayed for zero-*trans* influx of 500 μM [^{3}H]uridine (1 cpm/pmol) as described under Experimental procedures. The two sets of values at No NBTI represent two independent determinations of v_{12}^{zt}, and the average at each cell density (control) defines 100%.

Cell type	Growth stage (cells/ml)	Cell volume (μl/10^7 cells)	Uridine influx (v_{12}^{zt})					
			No NBTI		50 nM NBTI		500 nM NBTI	
			μM/s		μM/s	% of control	μM/s	% of control
CHO	$5 \cdot 10^5$	19.7	33.2 ± 4.2;	28.3 ± 2.6	9.8 ± 0.6	32	8.3 ± 0.6	27
	$7 \cdot 10^5$	18.1	16.0 ± 1.1;	17.2 ± 0.7	6.5 ± 0.6	39	6.2 ± 0.2	37
	$1 \cdot 10^6$	17.8	21.2 ± 0.8;	16.7 ± 0.4	7.2 ± 0.4	38	6.9 ± 0.3	36
	$1.4 \cdot 10^6$	17.4	21.3 ± 0.8;	17.8 ± 0.6	8.1 ± 0.7	41	6.2 ± 0.4	31
P388	$3.8 \cdot 10^5$	11.0	23.5 ± 2.0;	24.3 ± 1.5	3.7 ± 0.3	15	3.3 ± 0.2	13
	$1.2 \cdot 10^6$	9.2	23.2 ± 1.0;	25.1 ± 0.6	3.1 ± 0.1	12	2.6 ± 0.2	10
	$1.5 \cdot 10^6$	8.5	32.3 ± 1.8;	36.1 ± 2.3	4.1 ± 0.2	11	3.4 ± 0.2	9
	$1.8 \cdot 10^6$	6.8	28.7 ± 1.5	–	3.8 ± 0.3	13	3.2 ± 0.2	11

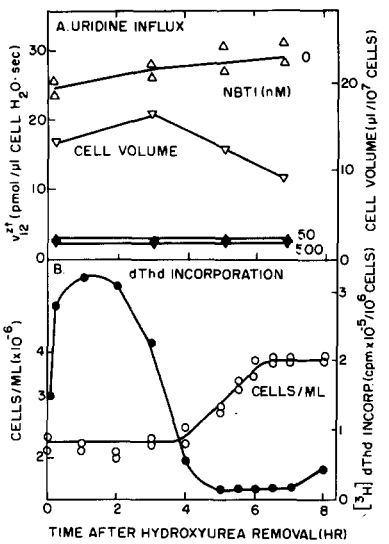

Fig. 3. Uridine influx in P388 cells as a function of cell cycle stage. A culture of $9 \cdot 10^5$ P388 cells/ml was supplemented with 0.5 mM hydroxyurea. After 10 h of incubation at 37°C (at $1.2 \cdot 10^6$ cells/ml), the cells were collected by centrifugation and suspended to the same density in fresh growth medium. After another 7 h of incubation at 37°C, the culture was again supplemented with 0.5 mM hydroxyurea and the cells harvested 7 h later (at $2 \cdot 10^6$ cells/ml). The cells were suspended in fresh growth medium (time zero), incubated at 37°C and analyzed as follows: The culture was monitored for cell density and samples thereof were incubated with 0.1 μM [^{3}H]thymidine (16 cpm/nmol) at 37°C for 15 min and then analyzed for radioactivity in acid-insoluble material (B). In addition, at the indicated times an about equal number of cells was collected by centrifugation and suspended to about $1.8 \cdot 10^7$ cells/ml of BM42B and assayed for zero-*trans* influx of 500 μM [^{3}H]uridine in the absence and presence of 50 or 500 nM NBTI at 25°C as described under Experimental procedures (A).

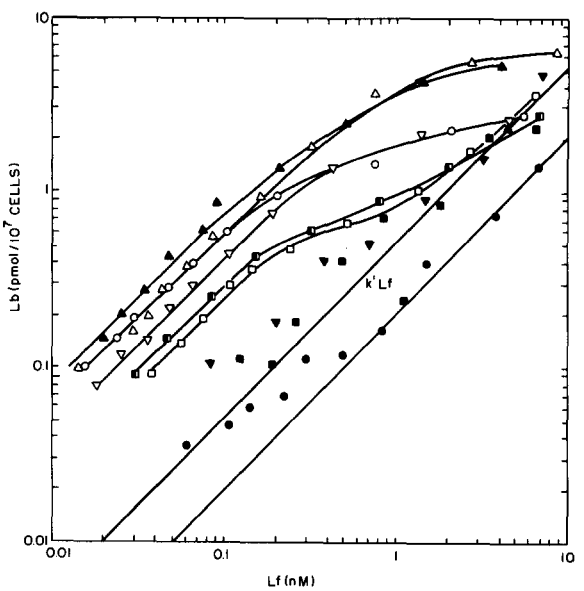

Fig. 4. Amounts of [^{3}H]NBTI bound (L_b) at equilibrium by various cell lines as a function of concentration of free NBTI (L_f). The equilibrium binding of [^{3}H]NBTI at concentrations ranging from 0.1 to 40 nM and at 3 μM was determined as described under Experimental procedures. Eqn. 1 was fitted to the data and the best-fitting parameters are listed in Table II. ●——●, ▼——▼, N1S1-67 wild type; ■——■, N1S1-67 (1-22); □——□, N1S1-67 (1-14-7); ○——○, P388; △——△, L929-2; ▲——▲, CHO wild type; ▽——▽, L1210; ◧——◧, S49. The area delineated by the lower two lines indicates the range of non-specific binding of NBTI as estimated by k' (see Table II) for P388, L929-2, CHO and L1210 cells.

ity of the cells (see later). Non-specific binding represented between 2 and 10% of the total binding at free NBTI concentrations below 0.5 nM.

In contrast to the results with P388, L929-2, CHO, L1210 and S49 cells, little, if any, high-affinity NBTI binding was detectable in wild-type and 1-22 Novikoff cells. The binding observed in wild-type Novikoff cells was non-saturable and resembled in magnitude that observed in the other cell lines (Fig. 4). Some high-affinity binding of NBTI, on the other hand, was detected in four experiments in the uridine kinase-deficient variant of Novikoff cells. The number of NBTI-binding sites/cell varied somewhat for different batches of these cells (the two extremes are presented in Table II), but was always only between 10 and 30% of that found in CHO and L929-2 cells which are of comparable size. The presence of a low number of high-affinity binding sites in uridine kinase-deficient Novikoff cells correlated well with the partial inhibition of uridine influx by low NBTI concentrations in these cells (Fig. 2A).

These results support the view that the inhibition of uridine transport by low concentrations of NBTI reflects its binding to high-affinity sites on the nucleoside transporter. Transporters without high-affinity binding sites are not inhibited by nmolar concentrations of NBTI. We have searched for other properties by which NBTI-sensitive and resistant nucleoside transporters can be distinguished, with limited success. The data in Figs. 1 and 2 already suggested that both NBTI-sensitive and resistant uridine transport are inhibited by dipyridamole and papaverine. This finding is

TABLE II

PARAMETERS FOR EQUILIBRIUM BINDING OF NBTI TO VARIOUS CELL LINES

Eqn. 1 was fitted to the data in Fig. 4 (Expt. 1) and those from additional experiments with each cell line (Expt. 2). k' as defined in Eqn. 1 is dimension-less; the values here are normalized to a cell density of 10^7 cells/ml, since non-specific binding is presumably proportional to cell density.

Cells	Intracellular space (μl/10^7 cells) [a]	Expt.	K_d (nM)	Binding sites per cell ($\times 10^{-5}$)	k' (ml/10^7 cells)
P388	9.9 ± 0.5 (17)	1	0.32 ± 0.04	1.4 ± 0.15	0.21 ± 0.010
		2	0.44 ± 0.04	3.4 ± 0.30	0.25 ± 0.030
L1210	13.2 ± 1.2 (10)	1	0.52 ± 0.63	2.1 ± 0.12	0.23 ± 0.023
		2	0.57 ± 0.16	2.6 ± 0.29	0.27 ± 0.019
CHO	21.3 ± 1.0 (30)	1	0.70 ± 0.53	4.2 ± 0.20	0.29 ± 0.020
		2	1.8 ± 0.45	5.7 ± 0.59	0.19 ± 0.033
L929-2	19.6 ± 2.0 (11)	1	1.0 ± 0.13	6.5 ± 0.60	0.25 ± 0.090
		2	1.0 ± 0.20	7.3 ± 0.62	0.52 ± 0.040
S49	5.9 (2)	1	0.37 ± 0.11	0.73 ± 0.06	0.10 ± 0.005
N1S1-67	25.4 ± 1.4 (29)	1	[b]	< 0.2	0.17 ± 0.006
		2	[b]	< 0.2	0.51 ± 0.023
N1S1-67 (1-22)		1	[b]	< 0.2	0.44 ± 0.029
N1S1-67 (1-14-7)		1	0.24 ± 0.06	0.48 ± 0.05	0.32 ± 0.058
		2	0.54 ± 0.10	1.90 ± 0.45	0.32 ± 0.020

[a] Mean ± S.E.M. of the number of experiments indicated in parenthesis.
[b] No specific binding was apparent.

documented more clearly in Table III. The presence of 100 nM NBTI reduced uridine transport in P388 cells to about 7% of control. The additional presence of 20 μM dipyridamole or 500 μM papaverine reduced it to less than 1% of control. The additional presence of mmolar concentrations of unlabeled uridine or of inosine also further reduced the influx of 500 μM [^{3}H]uridine to less than 1% of control, while hypoxanthine had a slight, but significant effect. An inhibition of

TABLE III

EFFECT OF VARIOUS SUBSTANCES ON NBTI-RESISTANT URIDINE TRANSPORT IN P388 CELLS

Samples of a suspension of $1.1 \cdot 10^7$ P388 cells/ml of BM42B were supplemented where indicated with 100 nM NBTI plus the indicated concentrations of other substances at 25°C. Then zero-*trans* influx of 320 μM [^{3}H]uridine (1.4 cpm/pmol) was measured as described under Experimental procedures.

NBTI (nM)	Other additions	μM	v_{12}^{zt}	
			pmol/μl cell water per s	% of control
0	None	0	17.1 ± 0.9	100
100	None	0	1.24 ± 0.1	7.3
100	Dipyridamole	20	0.060 ± 0.009	0.35
100	Papaverine	500	0.104 ± 0.020	0.60
100	Uridine	5000	0.150 ± 0.014	0.88
100	Inosine	2500	0.113 ± 0.016	0.66
100	Hypoxanthine	5000	0.94 ± 0.025	5.5

NBTI-resistant Urd influx by other nucleosides has also been reported for L1210 cells [14] and indicates that the NBTI-resistant component reflects a saturable transport process of broad specificity. The finding that total and NBTI-resistant uridine influx in L1210 cells were inhibited to a similar extent by various nucleosides [14] suggests that NBTI-sensitive and resistant Urd transport exhibits similar substrate specificity. This conclusion is supported by our finding that the NBTI-resistant and NBTI-sensitive zero-*trans* influx of 500 μM uridine in Novikoff and P388 cells, respectively, were about equally inhibited by 1 mM inosine, thymidine, cytidine and deoxycytidine and 100 μM 5'-deoxyadenosine (data not shown). Furthermore, the temperature dependence of NBTI-sensitive uridine transport in P388 cells ($E_a = 21$ kcal/mol) estimated from an Arrhenius plot of v^{ee} at 500 μM uridine between 5 and 32°C (data not shown) was also similar to that for NBTI-resistant nucleoside transport in Novikoff cells ($E_a = 18.3$ kcal/mol; Ref. 25).

We have shown previously that nucleoside transport in Novikoff cells is strongly inhibited by hypoxanthine and, vice versa, hypoxanthine transport is inhibited by nucleosides (see Ref. 1). The dose-response curves in Fig. 5, however, show that uridine transport in P388, L1210, L929-2 and CHO cells was far less inhibited by hypoxanthine than in wild-type Novikoff cells and its enzyme-deficient variants. The differential sensitivity of uridine transport in the various cell lines to hypoxanthine, however, seemed to be unrelated to the NBTI inhibitability. As shown in Table IV, the NBTI-resistant uridine transport in CHO cells was inhibited to a similar degree by hypoxanthine as total uridine transport in these cells. Results from another experiment indicated that the same is true for uridine transport in P388 and L1210 cells, but because of the low level of NBTI-resistant uridine transport in these cells, this correspondence was more difficult to establish unequivocally than in the case of CHO cells.

The effect of sulfhydryl reagents on nucleoside transport in mammalian cells has been somewhat

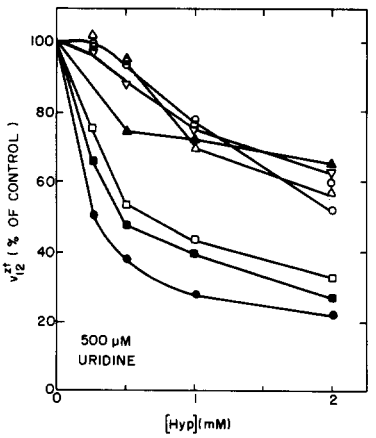

Fig. 5. Zero-*trans* influx of uridine by various cell lines as a function of concentration of hypoxanthine (Hyp). The uptake of 500 μM [^{3}H]uridine (0.8 cpm/pmol) was measured as described under Experimental procedures and illustrated in Fig. 1. The indicated concentrations of hypoxanthine were added simultaneously with substrate. ●———●, N1S1-67 wild type; ■———■, N1S1-67 (1-22); □———□, N1S1-67 (1-14-7); ○———○, P388; △———△, L929-2; ▲———▲, CHO wild type; ▽———▽, L1210.

TABLE IV

EFFECT OF HYPOXANTHINE ON TOTAL AND NBTI-RESISTANT URIDINE TRANSPORT IN CHO CELLS

In each experiment one portion of a suspension of CHO cells was supplemented with 1 μM NBTI and another portion did not receive NBTI. Then the zero-*trans* influx of 500 μM [^{3}H]uridine (1.1 cpm/pmol) was measured in the presence of the indicated concentrations of unlabeled hypoxanthine added simultaneously with substrate.

Hypoxanthine (mM)	No NBTI		+1 μM NBTI	
	v^{zt} (pmol/μl cell water per s)	%	v^{zt} (pmol/μl cell water per s)	%
0	22.9±5.1	100	5.5±0.4	100
0.5	19.9±3.4	87	5.0±0.5	91
1	15.8±1.6	69	3.9±0.4	71
2	11.5±0.9	50	2.7±0.2	49

controversial, though not explored in great detail. Some studies have shown that incubation with *p*-hydroxymercuribenzoate or pCMBS causes a marked inhibition of uridine uptake in a variety of cultured cells, whereas other reports indicated that low concentrations of pCMBS slightly stimulated uridine uptake in Golden hamster embryo cells (for summary, see Ref. 1). Under the conditions of these uptake measurements, however, substantial uridine metabolism occurred, making an unequivocal distinction between effects on transport and phosphorylation difficult [1]. In some reports, indirect evidence suggested that the transport step rather than uridine phosphorylation was inhibited by sulfhydryl reagents [26], and such inhibition of uridine transport per se has been confirmed for Novikoff cells [15]. Incubation of uridine kinase-deficient Novikoff cells with 50 μM *p*-hydroxymercuribenzoate at 37°C for 10 min greatly reduced uridine influx in these cells. In contrast, Belt [13,14] reported that uridine influx in wild-type and uridine kinase-deficient L1210 cells was little affected by incubation of the cells with pCMBS at 20°C for 20 min ($IC_{50} > 400$ μM), except for the small NBTI-resistant component (about 20% of total) which was strongly inhibited ($IC_{50} < 25$ μM). Her results suggested that sensitivity to inhibition by sulfhydryl reagents might be a specific property of NBTI-resistant uridine transport. Our present results obtained by incubating cells with sulfhydryl reagents at 37°C for 20 min do not agree with such a conclusion. First, we found no relationship between sulfhydryl reagent sensitivity of total uridine transport of the various cell lines and its NBTI inhibitability. Uridine transport was inhibited by *p*-hydroxymercuribenzoate in all cell lines examined, but to slightly different extents (Fig. 6). Total uridine transport in CHO cells, which is largely inhibitable by NBTI, was inhibited to the greatest extent by *p*-hydroxymercuribenzoate. On the other hand, the dose-response curve for NBTI-resistant uridine transport in Novikoff cells was similar to that for NBTI-sensitive uridine transport in P388, L1210 and L929-2 cells.

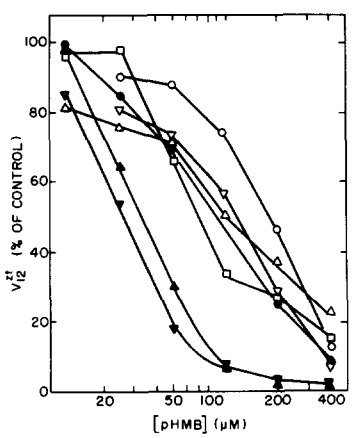

Fig. 6. Effect of preincubation of cells with various concentrations of *p*-hydroxymercuribenzoate (pHMB) on zero-*trans* uridine influx in a number of cell lines. Samples of suspensions of each cell line were incubated with the indicated concentrations of *p*-hydroxymercuribenzoate at 37°C for 30 min. Then the suspensions were equilibrated to 25°C and the zero-*trans* influx of 500 μM [^{3}H]uridine was measured as described under Experimental procedures. ●———●, N1S1-67 wild type; □———□, N1S1-67 (1-14-7); ○———○, P388; △———△, L929-2; ▲———▲, CHO wild type; ▼———▼, CHO-DAP-12; ▽———▽, L1210.

Second, the residual NBTI-resistant component of uridine transport in L1210 and CHO cells was indistinguishable in its sensitivity to *p*-hydroxymercuribenzoate from total uridine transport (Table V). This conclusion is derived from results obtained by two experimental approaches. In one experiment one half of a suspension of L1210 cells was treated with *p*-hydroxymercuribenzoate, while the other half was untreated. Then samples of each suspension were supplemented with various concentrations of NBTI and assayed for uridine influx. In the other experiment samples of a suspension of CHO cells were incubated with various concentrations of *p*-hydroxymercuribenzoate. Then each suspension was divided into halves; one half was supplemented with 1 μM NBTI, while the other half was not, and both were assayed for uridine influx. The results from both experiments supported equally the conclusion that *p*-hydroxymercuribenzoate sensitivity of uridine transport is unrelated to NBTI inhibitability. Results from other experiments extended this conclusion to inhibition of uridine influx by pCMBS in L1210 cells (data not shown).

In terms of substrate specificity and sensitivity to inhibition by dipyridamole, papaverine, and sulfhydryl reagents NBTI-sensitive and NBTI-resistant forms of nucleoside transport appear indis-

TABLE V

COMBINED EFFECTS OF NBTI AND *p*-HYDROXYMERCURIBENZOATE (pHMB) ON URIDINE TRANSPORT IN L1210 AND CHO CELLS

One portion of a suspension of $8 \cdot 10^6$ L1210 cells/ml was supplemented with 300 μm *p*-hyroxymercuribenzoate. This suspension and another portion without *p*-hydroxymercuribenzoate were incubated at 37°C for 30 min and then equilibrated at 25°C. Samples of each suspension were mixed with the indicated concentrations of NBTI and then assayed for zero-*trans* influx of 500 μM [^{3}H]uridine (0.6 cpm/pmol). Samples of a suspension of $1 \cdot 10^7$ CHO cells/ml were incubated with the indicated concentrations of *p*-hydroxymercuribenzoate at 37°C for 30 min. Then portions of each suspension were assayed for zero-*trans* influx of 500 μM [^{3}H]uridine (0.6 cpm/pmol) in the absence or the presence of 1 μM NBTI. v_{12}^{zt} in pmol/μl cell water per s. n.d., not determined.

Cells	NBTI (μM)	No pHMB		+300 μM pHMB	
		v_{12}^{zt}	%	v_{12}^{zt}	%
L1210	0	24.2 ±1.3	100	4.0 ±0.17	100
	0.5	20.3 ±1.1	83	3.3 ±0.14	82
	5	n.d.		1.0 ±0.08	25
	50	3.2 ±0.2	13	0.39±0.04	10
	500	2.8 ±0.2	12	0.32±0.03	8
Cells	pHMB (μM)	No NBTI		+1 μM NBTI	
		v_{12}^{zt}	%	v_{12}^{zt}	%
CHO	0	26.5 ±0.9	100	10.2 ±1.2	100
	20	17.8 ±0.8	67	6.5 ±0.7	64
	50	3.1 ±0.3	12	0.8 ±0.03	8
	100	0.43±0.05	1.6	0.18±0.02	1.6

tinguishable. In terms of the kinetic characters they are also similar as can be seen in Table VI. Michaelis-Menten parameters for nucleoside transport in Novikoff cells and in the predominantly NBTI-sensitive P388, L-67, S49 and HeLa cells have been reported previously [1,27], and are included in Table VI. Michaelis-Menten parameters for uridine transport in ATP-depleted, predominantly NBTI-sensitive L929 and L1210 cells have been measured in this study, and are reported there. The differences in K among the different cell types are not great and do not suggest any correlation between a transport system's inhibitability by NBTI and its affinity for uridine.

TABLE VI

KINETIC PARAMETERS FOR URIDINE TRANSPORT IN VARIOUS MAMMALIAN CELL LINES

The data for Novikoff cells and ATP-depleted P388, L-67, S49 and HeLa cells have been reported previously [1,24]. The kinetic parameters for uridine transport by L929 and L1210 cells were estimated in the same manner as summarized under Experimental procedures with cells depleted by ATP by incubation in glucose-free medium containing 5 mM KCN and 5 mM iodoacetate at 37°C for 10 min [14]. Zero-*trans* influx of 30, 60, 120, 240, 480, 960 and 1920 μM [^{3}H]uridine (480 cpm/ml, irrespective of concentration) was measured.

Cell line	K (μM)	V (pmol/μl cell water per s)
Novikoff	250±13 (12)	25.0±4
P388	230±17 (1)	19.2±1.7
L-67	252±10 (1)	8.2±0.1
S49 [a]	161±13 (1)	37.0±1.0
HeLa	187± 5 (1)	13.4±0.1
L1210	245±10 (1)	24.0±0.33
L929-2	175± 8 (1)	31.6±0.60

[a] Values are for thymidine rather than uridine transport.

CHO cells represent an interesting case where the NBTI-resistant transport component is sufficiently large as to permit a reasonably accurate measure of influx in the presence of NBTI, and would seem to afford an opportunity to evaluate the kinetics of NBTI-sensitive and -insensitive transport in the same cell population. The results of such an experiment are presented in Table VII. The obvious experimental design, however, is flawed, and the results presented must be regarded as approximations. In the first instance, kinetic measurements in the absence of NBTI contain contributions from the two forms of transporter, which cannot, a priori, be assumed to have identical parameters. In our analysis of the data, however, two components were not apparent, and the

TABLE VII

KINETICS OF URIDINE (Urd) TRANSPORT IN ATP-DEPLETED CHO CELLS WITH AND WITHOUT NBTI

The experiments were carried out as described in Table VI, except that a portion of the ATP-depleted cell suspension was incubated 5 min at 25°C with 500 nM NBTI before transport assay. The upper section of the table shows the computed initial velocities of transport at each concentration in Experiment 1. The lower section summarizes the Michaelis-Menten constants best fitting the data overall for Experiment 1 and a replicate experiment, for which individual velocities are not shown. v_{12}^{zt} in pmol/μl per s.

Expt. No.	Urd (μM)	Control v_{12}^{zt}	+500 nM NBTI v_{12}^{zt}	% of control
1	30	2.5	0.43	18
	60	4.1	0.78	19
	12	6.2	1.4	22
	24	8.3	2.1	25
	480	10.0	2.9	29
	960	11.1	3.6	32
	1920	11.8	4.1	35
1	K (μM)	123 ± 9	305 ±17	
	V (μM/s)	12.6± 0.3	4.8±0.1	
2	K (μM)	149 ±12	284 ±17	
	V (μM/s)	19.6± 0.6	6.6±0.1	

K and V shown ('control') were computed on a model that admits just one component. In the second instance, flux measurements in the presence of NBTI will not preclude contributions from the NBTI-sensitive component to the extent that high concentrations of uridine can displace NBTI from its high-affinity binding sites (see Ref. 7 and Introduction). Under the conditions employed, we believe this competitive relationship between NBTI and uridine is insignificant. Equating a Michaelis-Menten constant of about 250 μM with the dissociation constant for the uridine-carrier complex and taking the measured dissociation constant for the NBTI-carrier complex (Table II) allows estimation of the equilibrium among these three entities: at 500 μM total uridine and 500 nM total NBTI < 0.01% NBTI could be displaced from its binding site by uridine. Given these precautions, the K values shown in Table VII for CHO cells ± NBTI probably indicate a real difference in Michaelis-Menten constant in the two forms of uridine transport present in these cells.

One consequence of the higher K for the NBTI-insensitive transporter is an apparent increase in extent of NBTI inhibition as the concentration of transport substrate is lowered (Table VII). This consequence places an important, but not unmanageable, technical constraint on the assessment of partial inhibition by NBTI: the concentration of transport substrate should be high enough to approach maximum velocity, but not so high as to cause significant displacement of NBTI from its binding site.

Discussion

At least two forms of nucleoside transport have been identified in cultured mammalian cells on the basis of differential sensitivities to inhibition by NBTI and hypoxanthine. The system in Novikoff cells seems unique among the cell lines we investigated in its high resistance to NBTI and relatively high sensitivity to inhibition by hypoxanthine. Why the nucleoside transporter of Novikoff cells differs in its properties from those of other cell lines is not known. We are presently exploring whether these differences are related to the tissue (liver) or species (rat) origin of Novikoff cells. The finding that nucleoside transport in Walker 256 rat carcinoma cells also exhibits a high resistance to NBTI inhibition [14] suggest that the latter may be the case. In other cell lines with predominantly NBTI-sensitive nucleoside transport the proportion of total uridine transport that is NBTI-sensitive also differs in a manner which suggests possible species differences: about 90% in mouse cell lines, 60–70% in CHO cells (Fig. 2A) and 50–60% in HeLa cells [4,6]. The one exception is S49 mouse lymphoma cells which possess only NBTI sensitive uridine transport.

There is little doubt that the inhibition of nucleoside transport in the nmolar range (K_i = 0.1–1 nM) is a consequence of the interaction of NBTI with high-affinity sites (K_d = 0.3–1.8 nM, see Table II) on the transporters, although the nature of the high-affinity binding site remains to be elucidated. The enormous increment in affinity of sensitive nucleoside transporters for NBTI in comparison to natural nucleosides (approx. 10^5-fold) is entropy-driven [7] and presumably represents a hydrophobic association of the benzyl

moiety of NBTI with a site adjacent to, but distinct from, the nucleoside binding site of the carrier. The present study, and the results of Belt [13,14], indicate that the feature of the transporter system which participates in the hydrophobic interaction is absent from (or unavailable in) some transporters which are otherwise (with respect to substrate specificity, Michaelis-Menten constant, and sensitivity to several other inhibitors) quite similar to the NBTI-sensitive transporter.

While it cannot be rigorously excluded that the two types of transporter represent two independent gene products, it seems more plausible to us that both types are identical in primary structure, and that the hydrophobic interaction manifest in the one type resides in some sort of accessory component or in some conformational variation. At present, the nature of such a component or conformation is purely speculative: an additional membrane protein not crucially involved in nucleoside transport or a locally distinct phospholipid milieu would be candidates. An unambiguous test of this hypothesis may well have to await purification, reconstitution and structural analysis of the transporter(s). The appearance of a high-affinity NBTI-binding capability in the uridine kinase-deficient variant of Novikoff hepatoma cells seems more readily accommodated by the hypothesis.

In terms of this hypothesis, the results of our kinetic analysis of uridine transport in CHO cells with and without NBTI (Table VII) suggest that the presence of the presumptive accessory component is not totally without influence on the behavior of the transporter. The Michaelis-Menten constant with respect to uridine seems to be somewhat higher in its absence.

Aside from this apparent change in K_m in CHO cells, no other property investigated distinguishes NBTI-resistant and sensitive transport in a single cell line. They are indistinguishable in substrate specificity, sensitive to inhibition by dipyridamole, papaverine, sulfhydryl reagents, nucleoside analogs, and hypoxanthine, and in temperature dependence. Our results extend those reported by Belt [13,14] and are in general agreement with hers, except that we have not detected any difference in the sensitivity of NBTI-resistant and sensitive uridine transport to sulfhydryl reagents. The reason for its discrepancy is unknown. However, in other experiments we have shown that maximum or close to maximum inhibition of uridine influx in P388, CHO and Novikoff cells by a specific concentration of *p*-hydroxymercuribenzoate was attained during the first 10 to 20 min of incubation at 37°C and that transport inhibition correlated with the binding of *p*-hydroxymercuri[^{14}C]benzoate to the cells (Plagemann and Wohlhueter, submitted for publication). Furthermore, uridine transport in these cell lines was inhibited to about the same extent by preincubation with equivalent concentrations of *p*-hydroxymercuribenzoate and pCMBS.

Uridine transport in P388, L1210, CHO and L929-2 cells clearly differs in its sensitivity to inhibition by hypoxanthine from that in Novikoff cells (Fig. 6), but our data suggest that this difference applies also to the NBTI-resistant component of transport in these cells. At least in CHO cells where that information is, for technical reasons, most reliable, the hypoxanthine sensitivity of NBTI-resistant uridine transport seems identical to that of NBTI-sensitive transport. The mechanistic and physiological consequences of the difference in sensitivity to hypoxanthine is unclear, but, along with NBTI sensitivity, it is a property that differentiates multiple forms of nucleoside transport.

Our results demonstrate that a lack of high-affinity NBTI binding sites on cells does not prove the absence of functional nucleoside transporters, although the converse is probably true. The most convincing case is Novikoff cells which lack high-affinity NBTI binding sites, but transport nucleosides as efficiently as cells that possess high-affinity binding sites.

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