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Transport of Uridine and 6-Azauridine in Human Lymphoblastoid Cells

Specificity for the Uncharged 6-Azauridine Molecule

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SUMMARY

The transport of uridine and 6-azauridine was examined in the human lymphoblastoid cell line RPMI 6410/0 and a subline lacking uridine kinase activity. Uridine is transported with the same efficiency in both cells, with a K_T of 0.21 (± 0.02) mM and a V_{max} of 32 (± 4) pmoles/10^6 cells-sec. Examination of the effect of 6-azauridine on the initial rate of uridine uptake revealed that 6-azauridine (pK_a 6.7) is a very poor inhibitor of uridine transport, and that its inhibitory effect is strongly dependent upon pH. The apparent K_i is 14 (± 3) mM at pH 7.4, but decreases to 1.6 (± 0.2) mM at pH 6.0. In contrast, the K_T for uridine (pK_a 9.3) transport is the same at pH 7.4 and 6.0, suggesting that the nucleoside transporter itself is not directly affected by pH over this range. Direct measurements of the initial rate of uptake of 6-azauridine confirmed that its transport is pH-dependent, with the apparent K_T decreasing from 15.5 (± 3.8) mM at pH 7.4 to 3.6 (± 0.4) mM at pH 6.0 in the wild-type cells. Similar results were also obtained with the uridine kinase-deficient cells. The pH curve for the uptake of azauridine is similar to the dissociation curve of this nucleoside, with 50% of the uptake activity remaining at pH 6.7. These studies suggest that the nucleoside transporter of RPMI 6410 cells is not able to accommodate the anion of 6-azauridine and that uptake of this nucleoside occurs via transport of the undissociated molecule. Inhibition studies with 3-deazauridine (pK_a 6.5) at pH 7.4 and 6.0 indicate that the anion of this nucleoside, like that of 6-azauridine, does not inhibit uridine transport. On the other hand, inhibition of uridine transport by pyrazofurin (pK_a 6.7) is not affected by decreasing the pH to 6.0. These results suggest that structure and conformation, as well as the charge, may be important determinants in the ability of acidic nucleosides to bind to the nucleoside transporter.

INTRODUCTION

The uptake of physiological nucleosides and their cytotoxic analogues into mammalian cells is a complex process that involves the transport of the nucleoside across the plasma membrane followed by metabolism of the nucleoside within the cell. Through the use of rapid sampling techniques (2, 3), which permit accurate determination of the initial rate of uptake of radiolabeled nucleosides during the first few seconds, it has been possible to resolve the uptake process into its component parts of transport and metabolism (4, 5). These studies have demonstrated that the transport of most nucleosides remains rate-limiting for uptake only during the first few seconds of the uptake period; thereafter the intracellular nucleoside pool reaches a steady state, and phosphorylation of the nucleoside becomes rate-limiting.

Recent studies have shown that nucleoside transport is quite similar in a number of mammalian cells and occurs by a facilitated diffusion mechanism (4, 6) which is inhibited by NBMPR[1] and other S^6-derivatives of 6-thioinosine and 6-thioguanosine (6). This nucleoside transporter plays an important role in the uptake of all of the physiological nucleosides (4, 6) and many nucleoside drugs (5). Paterson *et al.* (5) have found that the transport inhibitor NBMPR protects certain cultured mammalian cells from more than 20 cytotoxic nucleoside analogues. It has also been shown that a nucleoside transport-deficient mutant of a murine lymphoma cell line is resistant to a number of toxic nucleosides (7). These studies demonstrate the importance of membrane transport in the action of nucleoside drugs and also illustrate the broad range of nucleosides that can be accommodated by this transporter. One of the questions that has been raised with respect to the specificity of this transporter for various nucleoside drugs is whether neg-

These studies were supported by National Cancer Institute Grant CA21677 and American Lebanese Syrian Associated Charities. A preliminary account of this work has been published (1).

[1] The abbreviation used is: NBMPR, nitrobenzylthioinosine, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine.

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atively charged nucleosides can be transported. Cass and Paterson (8) have previously observed that the acidic nucleosides 6-azauridine (pK_a 6.7) and orotidine (pK_a 2) do not stimulate the efflux of uridine from erythrocytes, and have suggested that charged nucleosides are not transported by this mechanism. More recent studies by Paterson *et al.* (9) have shown that the uptake of 6-azauridine (in 22- to 51-hr incubations) and its toxicity in human lymphoblastoid cells (RPMI 6410) are blocked by NBMPR. These results suggest that the uptake of 6-azauridine is mediated by the NBMPR-sensitive transporter, and again raises the question of whether negatively charged nucleosides are transported by this mechanism.

The present studies further examine the role of the NBMPR-sensitive nucleoside transporter in the uptake of acidic nucleosides. In order to probe the structural requirements of the transporter, we have examined the effect of pH on the transport of uridine (pK_a 9.3) and 6-azauridine (pK_a 6.7), and on the ability of neutral and acidic nucleosides to inhibit uridine transport in human lymphoblastoid cells. These studies have been carried out in a uridine kinase-deficient mutant, as well as the wild-type cells, to ensure that the observed effects can be attributed to transport and are not influenced by the intracellular phosphorylation of uridine or 6-azauridine.

MATERIALS AND METHODS

Cell lines and growth conditions. RPMI 6410 wild-type cells (RPMI 6410/0) and a subline (RPMI 6410/MP/DU) with less than 1% of the uridine kinase activity of the wild-type cells were provided by Dr. A. R. P. Paterson, University of Alberta. Since the RPMI 6410/MP/DU cells exhibited significant phosphorylation of 6-azauridine (10), another subline (RPMI 6410/MP/DU/AU) was cloned from a population of RPMI 6410/MP/DU selected for resistance to 1 mM 6-azauridine. This line, RPMI 6410/MP/DU/AU, does not phorphorylate 6-azauridine or 3-deazauridine (<0.1% of wild-type) (11). Both cell lines were free of mycoplasma.

The cells were grown as static suspension cultures in RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum at 37° in a humidified, 5% CO_2-air atmosphere. Cells used in transport studies were harvested during log growth ($\approx 5 \times 10^5$ cells/ml). All cell culture materials were obtained from GIBCO Laboratories (Grand Island, N. Y.).

Nucleoside transport assays. The transport of uridine was determined by a modification of the NBMPR-stop method described by Harley *et al.* (3). Cells were washed once and resuspended at 2×10^7 cells/ml in cold Buffer A [25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 130 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$]. The cell suspension was equilibrated at 22°, and 100 μl were layered over 100 μl of silicone oil (Versilube F-50 General Electric, Silicone Products Department, Waterford, N. Y.) in 1.5-ml centrifuge tubes. Uptake of uridine was initiated by rapidly adding 100 μl of a reaction mixture [Buffer A containing twice the indicated concentration of uridine and 0.5–1.0 μCi of [5-^{3}H]uridine (26 Ci/mmole); New England Nuclear Corporation (Boston, Mass.)] and terminated by adding 200 μl of Buffer A containing 20 μM NBMPR. The tubes were immediately centrifuged for 30 sec in a Beckman Microfuge B equipped with an Eppendorf 12-place 45° fixed-angle rotor. For measurements at pH 6.0, the cells were washed in Buffer A and then resuspended in Buffer B [25 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.0), 130 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$]; the reaction mixture and stopping solution were made with Buffer B. After all assays were completed, the supernatant fractions were aspirated and the upper portion of each tube (above the oil) was rinsed three times with water. The oil was removed with the last wash, and the cell pellet was dissolved in 0.5 ml of 1% Triton X-100 and counted in 5 ml of ACS scintillation fluid (Amersham Corporation, Arlington Heights, Ill.).

Uptake values at "zero-time" were determined by adding the stopping solution to the cells before the reaction mixture, and were subtracted from the values obtained at each time point. These "zero-time" values were independent of the uridine concentration and corresponded to the amount of extracellular fluid carried over into the cell pellet (determined from the carry-over of [^{14}C]sucrose into the pellet under similar conditions). The initial rates of uridine uptake were determined from the coefficients of the first-order terms in parabolas fit to the uptake data as described by Harley *et al.* (3). In some experiments initial rates were estimated from uptake during intervals of 5 sec rather than from complete time-courses. There were no significant differences between the kinetic constants determined by the two methods, indicating that uptake during an interval of 5 sec is a satisfactory estimate of the initial rate of uridine transport in these cells.

Transport of [5-^{3}H]6-azauridine (25 Ci/mmole; Moravek Biochemicals, Brea, Calif.) was determined in the manner described for uridine except that a 50-sec time course was used (unless otherwise indicated), and the initial rates were determined by linear regression analysis.

Other chemicals. NBMPR (12) was prepared from 6-thioinosine by Dr. Josef Nemec in this department. 6-Thioinosine and 3-deazauridine were generously provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.; pyrazofurin by Eli Lilly (Indianapolis, Ind.); and dipyridamole by Boehringer-Ingelheim (Ridgefield, Conn.). 5-Mercaptouridine and 6-hydroxyuridine were gifts from Dr. T. J. Bardos (State University of New York at Buffalo) and Dr. T. Ueda (Hokkaido University, Japan), respectively. All other nucleosides were obtained from Calbiochem-Behring Corporation (La Jolla, Calif.).

RESULTS AND DISCUSSION

Transport of uridine. Since the transport of nucleosides into mammalian cells is very rapid and intracellular phosphorylation of the nucleoside can become rate-limiting within a few seconds, uridine uptake measurements were made over a 10-sec time course. Uridine uptake by human lymphoblastoid cells (RPMI 6410/0) and a uridine kinase-deficient mutant (RPMI 6410/MP/DU/AU) at pH 7.4 are compared in Fig. 1. The initial phase of uptake in both cell lines is linear for only a few seconds.

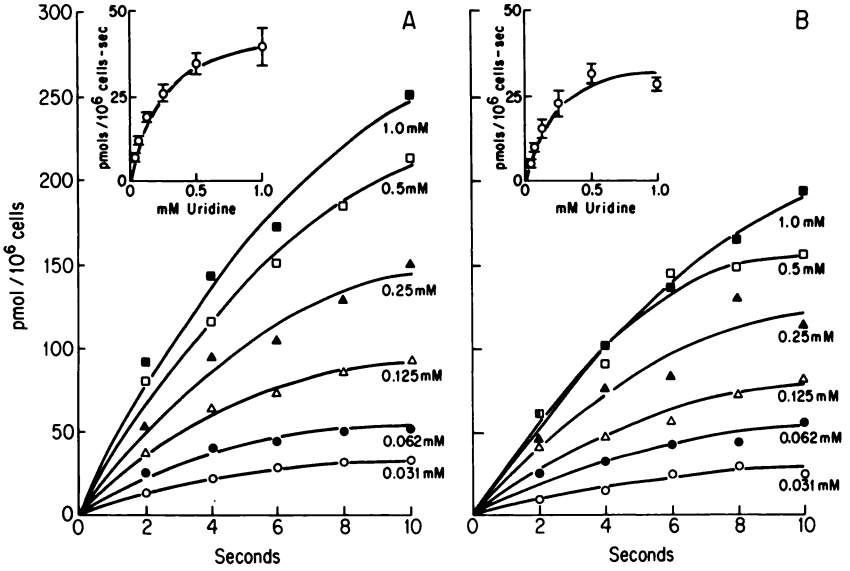

FIG. 1. *Uridine transport in RPMI 6410/MP/DU/AU and RPMI 6410/0 cells at pH 7.4*

Uridine uptake was determined over 10-sec time courses, and the initial rates were calculated from the fitted parabolas as described under Materials and Methods. The initial rates of uptake are replotted (± standard deviation) against the uridine concentration in the *insets*. The K_T and V_{max} were determined by direct computer fit of the hyperbolic rate equation as described by Cleland (13). Representative experiments for RPMI 6410/MP/DU/AU (A), and RPMI 6410/0 (B) are shown. The kinetic constants determined from these and several other experiments at pH 7.4 and 6.0 are summarized in Table 1.

Replots of the initial rates (Fig. 1, *insets*) reveal typical saturation kinetics with similar K_T and V_{max} values for both cell lines. The results of several experiments are summarized in Table 1. The close agreement of the kinetic constants between the two cell lines indicates that, under the conditions used in these assays, the transport rates determined in the wild-type cells are not influenced by the intracellular phosphorylation of uridine. It can also be seen in Table 1 that the K_T and V_{max} for uridine transport are not affected by lowering the pH to 6.0. The kinetic constants observed here are also similar to those reported for uridine transport in a number of other cultured mammalian cells (14–16).

Uridine transport in the RPMI 6410 cells also resembles that of other cell lines in its sensitivity to the nucleoside transport inhibitor NBMPR (5). The initial rate of uridine (100 μM) uptake is inhibited 50% at 2×10^{-9} M NBMPR, and greater than 95% inhibition is observed at 10^{-7} M NBMPR. Transport is also inhibited by dipyridamole ($IC_{50} \approx 2 \times 10^{-8}$ M), a less specific inhibitor of nucleoside transport (4).

Inhibition of uridine transport by azauridine. Paterson *et al.* (9) have previously shown that NBMPR protects RPMI 6410/0 cells from the toxic effects of azauridine (3 μM) and inhibits the uptake of [5-^{3}H]-6-azauridine in long-term incubations (22–51 hr), suggesting that azauridine enters these cells via the NBMPR-sensitive nucleoside transporter. Thus, it would be expected that as an alternate substrate for the transporter azauridine would inhibit uridine transport. As shown in Fig. 2, azauridine is a competitive inhibitor of the transport of uridine, although very high concentrations are required, and the K_i is 14 (± 3) mM at pH 7.4. The K_i decreases to 1.6 (± 0.2) mM at pH 6.0, whereas uridine transport itself is not affected by pH (Fig. 2; Table 1). At both pH values, azauridine is a simple linear-competitive inhibitor of uridine transport.

These results suggest that the nucleoside transporter is not directly influenced by pH, but rather that the transporter cannot accommodate the azauridine anion (pK_a 6.7), which predominates at pH 7.4. In fact, if the K_i values for azauridine inhibition of uridine transport are expressed in terms of the concentration of the neutral molecule, there is little difference between the values at pH 7.4 and 6.0 (2.4 mM and 1.4 mM, respectively). It should be noted that even when expressed in terms of the neutral molecule the K_i for azauridine is still very high as compared with the K_T of the transporter for uridine (0.2 mM), and azauridine therefore appears to have weak affinity for this transporter. The question of how efficient the transporter is for azauridine was examined further through direct measurement of the uptake of [5-^{3}H]6-azauridine.

Transport of azauridine. In contrast to the rapid fall-off in the rate of uridine uptake with time (Fig. 1), azauridine uptake is linear for at least 50 sec in both the wild-type and uridine kinase-deficient cell lines at both pH 7.4 and 6.0 (data shown for RPMI 6410/MP/DU/AU at pH 6.0 in Fig. 3). As with uridine, typical saturation

TABLE 1

Uridine transport in human lymphoblastoid cells

Initial rates of uridine uptake were determined as described under Materials and Methods, and the K_T and V_{max} values were calculated as described in Fig. 1. The number of experiments is indicated in parentheses.

Cell line	pH	K_T	V_{max}
		mM	*pmoles/10^6 cells-sec*
RPMI 6410/0 (9)	7.4	0.21 ± 0.02[a]	31 ± 4[a]
RPMI 6410/MP/DU/AU (3)	7.4	0.21 ± 0.04[a]	35 ± 9[a]
RPMI 6410/0 (2)	6.0	0.22 ± 0.02[b]	34 ± 8[b]

[a] Mean ± standard error of the mean.
[b] Mean ± one-half range.

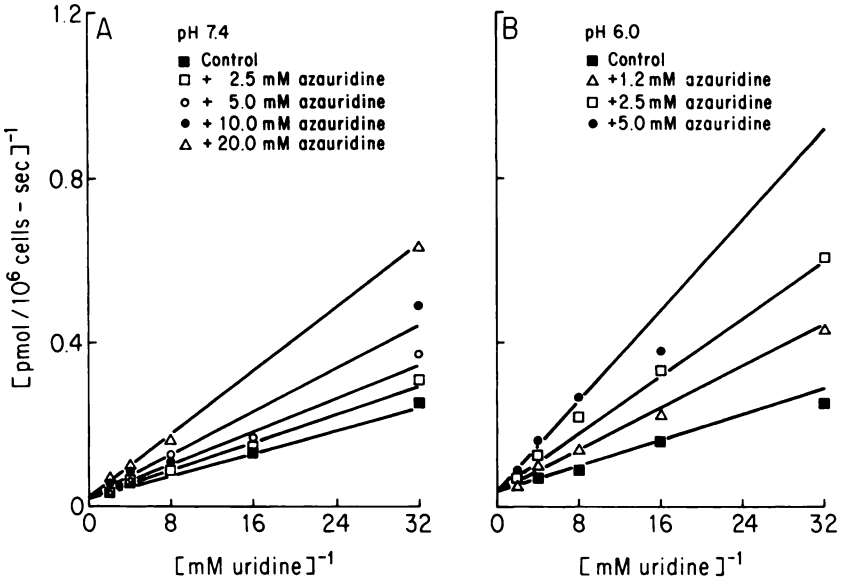

FIG. 2. *Inhibition of uridine transport by azauridine in RPMI 6410/0 cells at pH 7.4 and 6.0*

Uridine transport was determined over 5-sec intervals at five concentrations of uridine in the presence of the indicated concentrations of azauridine. Osmolarity was maintained by decreasing the NaCl concentration of the assay buffer. The plotted lines and the inhibition constants were determined by computer fit of the rate equation for competitive inhibition (13). The data did not fit the rate equation for noncompetitive inhibition. The mean (± one-half range) values for the inhibition constants determined in two experiments were as follows: (a) pH 7.4, 14 ± 3 mM, and (b) pH 6.0, 1.6 ± 0.2 mM.

kinetics are observed; however, extremely high concentrations of azauridine are required (*inset*, Fig. 3). The kinetic constants for azauridine transport in both cell lines are summarized in Table 2. There is no major difference in the transport of azauridine between wild-type and uridine kinase-deficient cells at either pH 6.0 or pH 7.4; however, the apparent K_T in both cell lines decreases 3- to 4-fold between pH 7.4 and 6.0. On the other hand, if the K_T values are expressed in terms of the concentration of the neutral molecule rather than the total azauridine concentration, there is no significant difference between the K_T values at pH 7.4 (2.6 mM) and pH 6.0 (3.0 mM). Thus, both the inhibition studies and the direct measurements of azauridine transport support the hypothesis that the nucleoside transporter has a marked specificity for the uncharged azauridine molecule. The high K_T value for the neutral molecule as compared with the K_T for uridine (0.2 mM) confirms that even the uncharged azauridine molecule has poor affinity for this transporter.

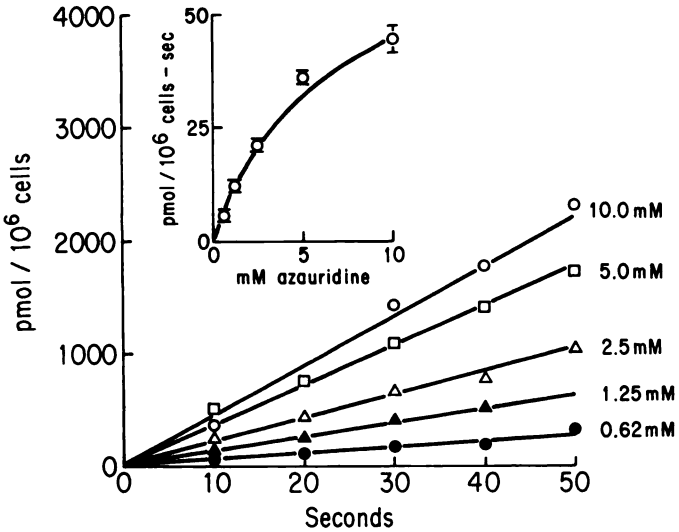

FIG. 3. *Azauridine transport in RPMI 6410/MP/DU/AU cells at pH 6.0*

Uptake was determined over 50-sec time courses at the indicated concentrations of azauridine. All assays were carried out in Buffer B, and osmolarity was maintained by decreasing the NaCl concentration. The initial rates of uptake were calculated by linear regression analysis and are replotted (± standard deviation) against the azauridine concentration in the *inset*. The K_T (5.5 ± 1.1 mM) and V_{max} (70.4 ± 6.9 pmoles/10^6 cells-sec) were determined by computer fit of the hyperbolic rate equation (13).

Uptake of azauridine over longer periods of time. The poor affinity of azauridine for the transporter and previous reports that azauridine is phosphorylated almost as efficiently as uridine by uridine kinase (17, 18) suggest that the transport of azauridine into the cell may be slower than its subsequent phosphorylation within the cell. As shown in Fig. 4, the uptake of azauridine (1 mM) by the wild-type cells remains linear for at least 10 min. Uptake is almost completely inhibited by 10 μM NBMPR and is inhibited about 75% by an equimolar concentration of uridine. The long period of linear uptake and the fact that all three lines extrapolate to the same point at 0 min are consistent with the hypothesis that transport, rather than phosphorylation, is rate-limiting to the uptake of this drug over an extended period of time. We have also observed that, when RPMI 6410/0 and a RPMI 6410/MP/DU/AU cells are incubated with azauridine (5 μM) for prolonged periods (6 hr under culture conditions), the intracellular level of free nucleoside in the wild-type cells is less than one-half that found in the uridine kinase-deficient cells,[2] again suggesting that the transport of

[2] J. A. Belt, G. S. Germain, and A. D. Welch, unpublished results.

TABLE 2

Azauridine transport in human lymphoblastoid cells

Initial rates of azauridine uptake were determined from 50-sec time courses as described under Materials and Methods. Osmolarity was held constant with increasing azauridine concentration by decreasing the NaCl. The K_T and V_{max} values were determined as in Fig. 1 and are reported as the mean ± standard error of the mean. The number of experiments used in the computer fit of the rate equation is indicated in parentheses.

Cell line	pH	K_T[a]	K'_T[b]	V_{max}
		mM	*mM*	*pmoles/10⁶ cells-sec*
RPMI 6410/0 (1)	7.4	15.5 ± 3.8	2.6	73 ± 10
RPMI 6410/MP/DU/AU (2)	7.4	14.8 ± 1.9	2.5	46 ± 3
RPMI 6410/0 (1)	6.0	3.6 ± 0.4	3.0	108 ± 5
RPMI 6410/MP/DU/AU (2)	6.0	5.4 ± 1.0	4.5	70 ± 5

[a] Expressed in terms of the total azauridine concentration.
[b] Expressed in terms of the concentration of the neutral molecule.

azauridine into these cells is not fast enough to keep up with its phosphorylation. This is unusual in that the transport of other nucleosides is considerably faster than their phosphorylation (4, 5).

The effect of pH on azauridine (5 mM) uptake is shown in Fig. 5. Uptake decreases steadily over the pH range from 5.5 to 8.0 and the shape of the curve is similar to the dissociation curve (*dashed line*) for azauridine, with 50% of the uptake activity remaining at pH 6.7. In contrast, the transport of uridine (0.1 mM) is not affected over this pH range (Fig. 5). Since transport appears to be the rate-limiting step in the uptake of azauridine, this pH curve probably reflects the effect of pH on the transport of azauridine. These results are consistent with the observed decreases in the apparent K_T and K_i values for azauridine at pH 6.0 as compared with pH 7.4, and further support the conclusion that the uptake of azauridine occurs via transport of the uncharged molecule.

Inhibition of uridine transport by other nucleosides at pH 7.4 and 6.0. The effect of several other nucleosides on uridine transport at pH 7.4 and 6.0 in RPMI 6410/0 cells is examined in Fig. 6. As would be expected if the nucleoside transporter itself is not directly affected by pH, inhibition of uridine transport by uncharged nucleosides (adenosine and thymidine) is the same at pH 7.4 and 6.0. In contrast, inhibition by 3-deazauridine (pK_a 6.5) (19) is strongly dependent upon pH, and the IC_{50} decreases from 5.8 mM at pH 7.4 to less than 1 mM at pH 6.0. More acidic nucleosides, 5-mercaptouridine (pK_a 5.0) and 6-hydroxyuridine (pK_a 3.75), do not inhibit uridine transport even at pH 6.0 (data not shown). Thus it appears that the anions of these acidic nucleosides, like that of azauridine, do not inhibit uridine transport.

Inhibition of uridine transport by pyrazofurin (pK_a 6.7) (20), on the other hand, is not affected by pH. Since the concentration of the neutral form of pyrazofurin increases 5-fold over this pH range, a change in the IC_{50} value should have been observed unless the anion, as well as the neutral molecule, is able to combine with the transporter to inhibit uridine transport. It is not readily apparent why the nucleoside transporter should be able to accommodate a negative charge on pyrazofurin but not on 6-azauridine or 3-deazauridine. Pyrazofurin is an unusual nucleoside analogue in that the glycosidic linkage is through a carbon-carbon bond rather than a carbon-

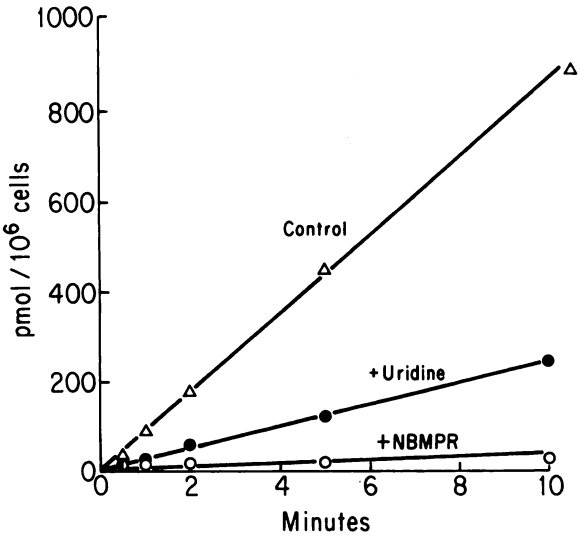

FIG. 4. *Azauridine uptake in RPMI 6410/0 cells at pH 7.4*

Azauridine uptake was determined over a 10-min time course as described under Materials and Methods, except that RPMI 1640 medium (without bicarbonate) supplemented with 25 mM Na-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) was substituted for Buffer A. The azauridine concentration was 1.0 mM, uridine 1.0 mM, and NBMPR 10 μM.

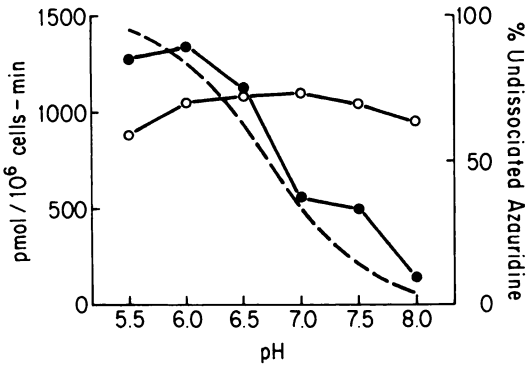

FIG. 5. *Effect of pH on azauridine and uridine uptake in RPMI 6410/0 cells*

Uptake assays were carried out as described under Materials and Methods, except that the cells were washed and resuspended in RPMI 1640 medium (without bicarbonate) supplemented with 25 mM Tris-2-(*N*-morpholino)ethanesulfonic acid (pH 7.0), and the uptake assays were in the same medium at the indicated pH. Uridine (0.1 mM) transport was determined over a 5-sec interval (○) and azauridine (5.0 mM) uptake over a 5-min interval (●). The results are presented as picomoles per 10^6 cells per minute for both substrates. The *dashed line* is the dissociation curve for azauridine.

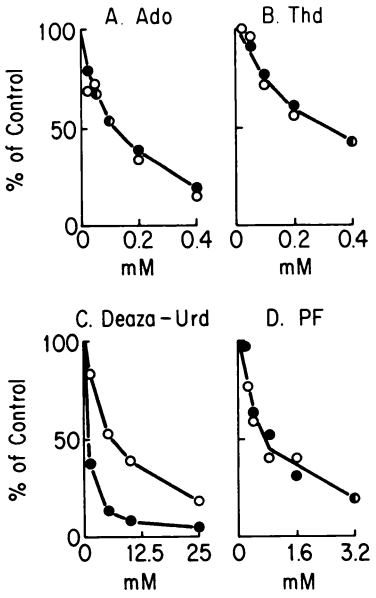

FIG. 6. *Inhibition of uridine transport in RPMI 6410/0 cells by other nucleosides at pH 7.4 and 6.0*

Uridine (100 μM) transport was determined over 5-sec intervals at pH 7.4 (○) and 6.0 (●) in the presence of the indicated concentrations of inhibitor (*Ado*, adenosine; *Thd*, thymidine; *Deaza-Urd*, 3-deazauridine; *PF*, pyrazofurin). In C, the osmolarity was maintained by decreasing the NaCl in the assay buffer.

nitrogen bond. This analogue exhibits biochemical properties of both purine and pyrimidine nucleosides. Its phosphorylation is catalyzed by adenosine kinase (21), and pyrazofurin monophosphate blocks pyrimidine biosynthesis through inhibition of orotidine monophosphate decarboxylase (22). Another group of *C*-nucleosides, the formycins, also exhibits properties of both purine and pyrimidine nucleosides, and these unusual biological effects have been attributed to increased conformational flexibility about the carbon-carbon glycosidic bond (23). If pyrazofurin also has increased conformational flexibility, the anion may be able to assume a conformation in which the negative charge can be accommodated by the nucleoside transporter.

CONCLUSIONS

In agreement with the original observation of Cass and Paterson (8), made in erythrocytes, it appears that the nucleoside transporter of RPMI 6410 cells is not able to accommodate the negative charge on the pyrimidine ring of azauridine at physiological pH. The uptake of azauridine that does occur at pH 7.4 appears to be via the transport of the undissociated molecule, which at this pH is about 20% of the total azauridine concentration. This limitation on nucleoside transport may also affect the uptake of 3-deazauridine and certain other acidic nucleosides, but it cannot be generalized to all acidic nucleosides as indicated by the lack of a pH effect on the inhibition of uridine transport by pyrazofurin. These results suggest that structure and conformation, as well as charge, may be important determinants in the ability of acidic nucleosides to bind to the nucleoside transporter. Whether this also implies a more complex structural or conformational specificity for the transport of acidic nucleosides must await further studies utilizing direct measurements of the transport of pyrazofurin and other acidic nucleoside analogues.

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