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STRUCTURAL MODIFICATIONS AT THE 2′- AND 3′-POSITIONS OF SOME PYRIMIDINE NUCLEOSIDES AS DETERMINANTS OF THEIR INTERACTION WITH THE MOUSE ERYTHROCYTE NUCLEOSIDE TRANSPORTER

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Abstract—Modifications in the sugar moiety of pyrimidine nucleosides may affect their ability to function as permeants of the mouse erythrocyte nucleoside transporter. In this investigation, a number of synthetic uracil and thymine nucleosides which differ from the physiological nucleosides, uridine, deoxyuridine and thymidine, through structural changes at the 2′- and 3′-positions were studied. Interaction of the analogs with the transporter has been assessed in terms of their affinities for an external site on the transporter as well as their abilities to effect *trans*-acceleration of thymidine efflux. 1-(β-D-Arabinofuranosyl) uracil (araU) and 1-(β-D-arabinofuranosyl)thymine (araT) were comparable to thymidine as permeants while nucleosides in which the 3′-hydroxyl was replaced with hydrogen or a halogen had a decreased affinity for the transporter. 3′-Fluoro-3′-deoxy-araU weakly accelerated thymidine efflux while its ribo-isomer and the other 3′-halogeno-3′deoxy-arabino analogs as well as dideoxythymidine inhibited efflux. The absence of 2′- and 3′-carbons in acyclothymidine and acyclouridine strongly decreased the affinities of these nucleosides for the transporter; efflux of thymidine was not accelerated in the presence of these compounds. The conformationally constrained cyclic nucleoside 2,2′-anhydro-araU had a very low affinity for the transporter, and influx of the radiolabeled compound could not be demonstrated. The results suggest that modification at the 3′-position, loss of a portion of the sugar ring, and lack of conformational flexibility are factors which decrease the abilities of some pyrimidine nucleosides to function as permeants. It is suggested that combined effects of substituents which play a role in determining nucleoside conformation should be considered in assessing structural requirements for permeants of the transporter.

Modifications in the molecular structure of the physiological sugar moiety (ribose or deoxyribose) of pyrimidine nucleosides have yielded compounds which are biologically active as antiviral [1, 2] or antitumor [3–6] drugs, or which are inhibitors of uridine phosphorylase (EC 2.4.2.3) [7]. The potential of 2′- and 3′-halogeno pyrimidine nucleosides as diagnostic radiopharmaceuticals has been investigated [8–10]. The design of nucleoside drugs which may have therapeutic or diagnostic value is greatly aided by an understanding of the mechanism by which existing compounds exert their effects *in vivo*. Since the activity of many of these compounds depends upon their entry into intracellular metabolic pathways, an ability to cross cell membranes is a requisite of their effectiveness. Thus, it is useful to examine the effects of structural modifications on membrane transportability.

Nucleoside transport across mammalian cell membranes occurs by a facilitated diffusion mechanism which accepts a wide variety of pyrimidine and purine nucleosides as substrates [11, 12]. Limitations on the structural variations tolerated by the transporter have been demonstrated previously. Modification in the sugar moiety has been shown to be more critical than that in the nucleobase [13, 14], the presence of a 3′-hydroxyl in the α-configuration has been shown to be an important determinant for purine nucleoside transport [15, 16], deoxyuridines having a halogen other than fluorine at the 2′-position have been found to be poor substrates for the transporter [17], and some negatively charged pyrimidine nucleosides are not accepted by the transporter [18].

In the present investigation, the interactions of some modified nucleosides of uracil and thymine with the nucleoside transport mechanism of mouse erythrocytes were studied. The erythrocyte transporter was chosen as a model because it has been well-characterized kinetically [19–21] and is known to have a broad permeant specificity [13, 14, 17]. Metabolism of thymidine does not occur in human [22] or mouse (this work) erythrocytes, so that these cells provide an uncomplicated transport model and allow the implementation of an important approach to the identification of non-radioactive substances as permeants, namely the study of *trans*-effects on the efflux from cells of radioactively labeled physiological nucleosides. *Trans*-effects are not demonstrable in some cultured mammalian cells [23].

In this study, we have attempted to evaluate the

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contributions of structural features of pyrimidine nucleosides to interactions with the transporter; in particular, configuration of the 2′-hydroxyl, replacement of the 3′-hydroxyl with a hydrogen or halogen, deletion of carbons 2′ and 3′, and conformational restrictions effected by a 2,2′-cyclization have been examined. Interaction of the modified nucleosides with the transporter was evaluated by (a) inhibitory effects on zero-*trans** influx of thymidine, and (b) *trans*-effects on efflux of thymidine. In the latter procedure, *trans*-acceleration of efflux provides evidence that the test compound is translocated across the membrane by the nucleoside transporter.

MATERIALS AND METHODS

Materials. Male B10D2F$_1$ mice (C57BL/10J × DBA/2J, F$_1$) were obtained from the Health Sciences Small Animal Program, University of Alberta. 3′-Fluoro-3′-deoxy-araU† and 3′-chloro-3′-deoxy-araU were prepared by literature methods. 3′-Fluoro-3′-deoxy-riboU was synthesized by a method which will be reported elsewhere.‡ 3′-Bromo-3′-deoxy-araU, 3′-iodo-3′-deoxy-araU, acyclouridine and acyclothymidine were provided by Dr. Y. W. Lee, Mr. J. R. Mercer and Mr. T. Iwashina. AraU, araT and 2′,3′-dideoxythymidine were purchased from Calbiochem-Behring, San Diego, CA. 2,2′-Anhydro-araU, thymidine, 2′-deoxyuridine and TES were purchased from the Sigma Chemical Co., St. Louis, MO. NBMPR was purchased from Terochem Laboratories Ltd., Edmonton, Alta. [6-^{3}H]Thymidine (20–30 Ci/mmole) and inulin[^{14}C]carboxylic acid (15.4 mCi/mmole) were purchased from the Amersham Corp., Oakville, Ontario. [2-^{14}C]2,2′-Anhydro-araU (70 mCi/mmole) was provided by Dr. L. Gati. Aquasol-2 liquid scintillation fluid was purchased from NEN Canada, Lachine, Quebec. Polyethyleneimine impregnated cellulose MN 300 thin-layer sheets with fluorescence indicator were purchased from Brinkman Instruments (Canada) Ltd., Rexdale, Ontario.

Male B10D2F$_1$ mice were asphyxiated with CO_2, and blood was collected into 3.8% citrate solution by cardiac puncture and used the same day. Mouse erythrocytes obtained by this procedure have yielded influx kinetic data for physiological pyrimidine nucleosides which are similar to literature values for mouse erythrocytes collected by a different procedure [24]. All centrifugations were performed in an Eppendorf microcentrifuge at 12,800 *g* for 1 min. Nucleoside flux measurements were conducted at 25° in a circulating water bath.

Investigation of thymidine metabolism in mouse erythrocytes. After removal of plasma and buffy coat, packed erythrocytes were washed with buffered saline§ and incubated at a 50% hematocrit with equilibrium concentrations of 0.5 or 5 mM [6-^{3}H] thymidine for 30 min at 37° followed by 3 hr at 25°. The pelleted cells were extracted with chilled 5% perchloric acid, and the extract was neutralized and chromatographed with carrier thymine, thymidine, and dTMP on polyethyleneimine-cellulose thin-layer sheets (pre-washed with 50% methanol solution) using 0.1 M LiCl/0.06 M $(NH_4)_2SO_4$ as solvent system [25]. The compounds were located by fluorescence quenching, eluted overnight in 0.7 M $MgCl_2$/0.02 M Tris–HCl, pH 7.4 [26], and counted in Aquasol-2 by liquid scintillation methods.

Transport experiments. The methods used have been described in detail previously [17]. The brief descriptions which follow include some modifications used in the present work.

For influx experiments, mouse erythrocytes were washed in buffered saline and suspended in the same medium at an 11% hematocrit. Nucleoside influx was initiated by the rapid addition of cell suspension to buffered saline containing [6-^{3}H]thymidine at four concentrations (0.05 to 0.5 mM) alone or together with various concentrations of test nucleosides. This provided a series of sixteen individual influx suspensions for each test nucleoside (except for 3′-chloro-3′-deoxy-araU where only twelve were used). Duplicate aliquots from each suspension were analyzed. The hematocrit during the influx period was 5%. After 3 sec, transport was terminated by the addition of NBMPR [27, 28] at a final concentration of 10 μM. Cells were pelleted and washed once with NBMPR solution. In separate experiments (not shown) we have determined that two further washings of the cells with NBMPR solution resulted in no loss of radioactivity from the cell pellet, indicating that intracellular nucleoside was not being removed by this procedure. Cell pellets were extracted with chilled 5% perchloric acid and, after at least 30 min at 4°, portions of the extracts were removed and counted in Aquasol-2 by liquid scintillation methods. Radioactivity trapped in the extracellular space of the pellet was determined in triplicate as a zero-time value, by reversal of the order of addition of NBMPR and nucleoside to the cells. This value was subtracted from radioactivity in the influx samples.

Zero-*trans* influx of 9 mM [2-^{14}C]2,2′-anhydro-araU was measured in 5% cell suspension at five time intervals from 3 sec to 5 min. NBMPR was added to terminate transport, but the washing step with NBMPR solution was omitted. Extraction of the cells with perchloric acid was performed as above, and radioactivity was determined in the extracts.

Efflux of [6-^{3}H]thymidine into solutions containing test nucleosides was determined in order to measure *trans*-effects of these substances. Cells were "loaded" with 5 mM [6-^{3}H]thymidine by incubation at 37° for

* Zero-*trans* fluxes refer to movement of nucleoside molecules from one side (*cis*) of the membrane to the other side (*trans*), where the concentration of nucleoside is assumed to be zero.

† Abbreviations: araU, 1-(β-D-arabinofuranosyl)uracil; araT, 1-(β-D-arabinofuranosyl)thymine; araC, 1-(β-D-arabinofuranosyl)cytosine; araA, 1-(β-D-arabinofuranosyl) adenine; riboU, uridine or 1-(β-D-ribofuranosyl)uracil; acyclouridine, 1-(2-hydroxyethoxymethyl)uracil; acyclothymidine, 1-(2-hydroxyethoxymethyl)thymine; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid; and NBMPR, nitrobenzylthioinosine or 6-[(4-nitrobenzyl) thio]-9-β-D-ribofuranosylpurine.

‡ H. K. Misra, W. P. Gati, E. E. Knaus and L. I. Wiebe, *J. heterocycl. Chem.*, in press.

§ Buffered saline contained 140 mM NaCl, 1.4 mM $MgSO_4$ and 18 mM TES, at pH 7.4.

30 min at a 50% hematocrit in solutions containing 8.5 mM [6-^{3}H]thymidine. Following centrifugation at the end of the incubation period, the equilibrium concentration of thymidine was confirmed to be 5 mM by measurement of radioactivity in the extracellular medium. Efflux assays were initiated at 25° by rapidly suspending loaded cells at a 5% hematocrit in solutions of nucleoside in buffered saline with magnetic stirring. After intervals of 10 and 20 sec, NBMPR solution was added at a final concentration of 10 μM. Separate cell suspensions were used for different time points. Zero time points were determined by suspending the cells in NBMPR solution before addition of buffered saline. Concentrations of [6-^{3}H]thymidine in the extracellular medium were determined by liquid scintillation counting of portions of suspension supernatant fluid in Aquasol-2.

RESULTS

Incubation of mouse erythrocytes in the presence of 0.5 or 5 mM [6-^{3}H]thymidine under the conditions used to prepare the cells for efflux experiments showed that less than 3% of this substrate disappeared. This result is consistent with other reports of the lack of metabolism of either uridine or thymidine in human erythrocytes [22], and is a necessary condition in efflux experiments with thymidine which require that cells be loaded with this permeant.

Thymidine flux measurements are expressed in units of nmoles thymidine/10^{10} cells/sec. The cell water content of cell pellets was determined as the difference between the extracellular space (11%, determined as the inulin [^{14}C]carboxylic acid space) and the total moisture content of the cell pellets (70%, determined by drying to constant weight at 110° [22]). These measurements yielded a value of 310 μl cell water/10^{10} cells, under the conditions of these experiments.

Influx experiments. The concentration dependence of zero-*trans* influx of thymidine was plotted according to Hanes [29], yielding kinetic parameters (±S.D.) of $K_m = 0.098 \pm 0.001$ mM and $V_{max} = 7.5 \pm 0.1$ nmoles/10^{10} cells/sec. Analysis of these data according to Eisenthal and Cornish-Bowden [30] yielded values which were identical within the estimated error.

Competition of modified nucleosides with thymidine for entry at an external transporter site was demonstrated in influx competition experiments with [6-^{3}H]thymidine. The data were plotted according to Dixon [31]; examples are shown as insets in Fig. 1. For all compounds tested, the lines intersected at points above the [I]-axis. Assuming that these data

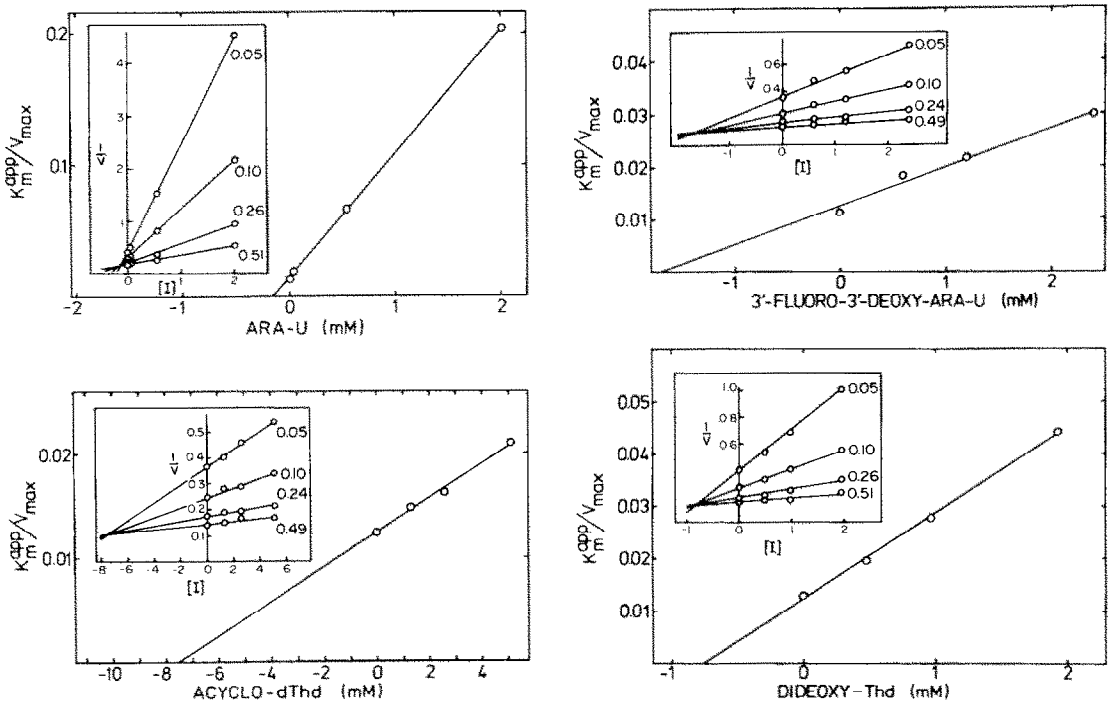
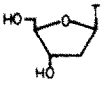
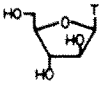
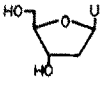
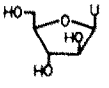
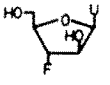
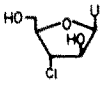
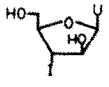
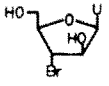
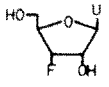
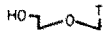
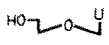
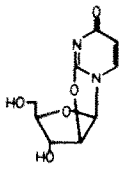


Fig. 1. Examples of plots used for determination of K_i values for modified nucleosides in influx competition experiments with thymidine. K_m^{app} and V_{max} values were determined at each inhibitor concentration according to Eisenthal and Cornish-Bowden [30]. Shown are replots of the ratios of these values as a function of [I] [32]. Units of the ordinate are mM·(nmoles thymidine/10^{10} cells/sec)$^{-1}$. The lines were fitted by linear regression analysis. Insets show examples of Dixon plots [31] of inhibition data. Each point represents the mean value of duplicate measurements from a single cell suspension. Units of the ordinate are (nmoles thymidine/10^{10} cells/sec)$^{-1}$, while those of the abscissa are mM. Thymidine concentrations (mM) are shown beside each line. The lines were fitted by a visual best fit method.

Table 1. Structurally modified nucleosides: their K_i values in influx competition with thymidine and their *trans*-effects on thymidine efflux

Compound	Structure*	$K_i \pm$ S.D. (mM)	Efflux of thymidine ± S.D. (nmoles/10^{10} cells/sec) from cells loaded with 5 mM thymidine into solutions containing test compounds at concentrations of:		
			0.5 mM	5 mM	10 mM
None (buffered saline)				7.8 ± 0.7	
Thymidine		0.098 ± 0.001 (K_m)	12 ± 1	15 ± 1	
AraT		0.15 ± 0.01	14 ± 1	15 ± 1	
Deoxyuridine		0.11 ± 0.02	13 ± 2	18 ± 2	
AraU		0.16 ± 0.01	16 ± 1	13 ± 1	
2′,3′-Dideoxythymidine		0.75 ± 0.05	5.6 ± 1.5	−1.6 ± 2.2	
3′-Fluoro-3′-deoxy-araU		1.7 ± 0.3	7.2 ± 1.0	9.5 ± 1.6	
3′-Chloro-3′-deoxy-araU		2.7 ± 1.4	7.5 ± 1.4	5.1 ± 0.9	
3′-Iodo-3′-deoxy-araU		3.0 ± 0.1	6.0 ± 1.2	1.5 ± 1.7	
3′-Bromo-3′-deoxy-araU		3.7 ± 0.2		6.5 ± 2.2	3.9 ± 1.8
3′-Fluoro-3′-deoxy-riboU		3.8 ± 1.2		4.2 ± 1.0	2.0 ± 1.6
Acyclothymidine		7.5 ± 0.7		5.5 ± 1.8	7.1 ± 0.7
Acyclouridine		11 ± 1		5.9 ± 1.3	5.4 ± 2.0
2,2′-Anhydro-araU		14 ± 4		ND†	

* T = thymine, U = uracil.
† ND = not determined.

represent competitive inhibition, K_i values were determined by plotting influx competition data according to Eisenthal and Cornish-Bowden [30] to obtain K_m^{app} and V_{max} values, and replotting the ratio of these parameters as a function of [I] [32].

The K_i values are given in Table 1. These reflect a wide range of affinities for the transporter. AraU and araT had affinities comparable to those of thymidine and deoxyuridine. The absence of a hydroxyl at the 3′-position of thymidine (dideoxythymidine) significantly increased the K_i value, indicating that this substituent plays a role in binding of pyrimidine nucleosides to the transporter.

The four 3′-halogeno-3′-deoxy-derivatives of araU had K_i values (1.7 to 3.7 mM) ranging from about ten to twenty times that of araU and two to five times that of dideoxythymidine, suggesting that replacement of the 3′-hydroxyl with a halogen is considerably less acceptable than replacement with a hydrogen, in terms of affinity for the transporter. Within this group of halogenated compounds, the K_i values of the bromo and iodo compounds were greater than that of the fluoro compound, suggesting that an increase in size of the halogen atom and/or a decrease in electronegativity would decrease affinity for the transporter. A second isomer of the 3′-fluoro-3′-deoxynucleoside of uracil in which the 2′-hydroxyl was in the ribo-configuration had a K_i value (3.8 mM) about twice as great as that of the arabino isomer.

The acyclic "nucleosides" of thymine and uracil, acyclothymidine and acyclouridine, had only a weak affinity for the transporter, with K_i values of 7.5 and 11 mM respectively. Of all compounds studied, that which showed the least affinity was 2,2′-anhydro-araU, with a K_i of 14 mM. No detectable zero-*trans* influx of the anhydro compound occurred during incubation of mouse erythrocytes with 9 mM [2-^{14}C] 2,2′-anhydro-araU for intervals of 3 sec to 5 min. There was no increase in cell-associated radioactivity as a function of time, and all radioactivity measurable in the cell extracts could be accounted for as labeled substrate present in the extracellular space of the cell pellets.

Efflux experiments. *Trans*-acceleration (accelerative exchange diffusion) of thymidine efflux by extracellular thymidine was investigated using cells loaded with 5 mM thymidine and a series of concentrations of thymidine in the suspending medium. Results are shown in Fig. 2. This process was saturable, and efflux into 5 mM thymidine reached a velocity (15 nmoles/10^{10} cells/sec) approximately twice as great as efflux into buffered saline (7.8 nmoles/10^{10} cells/sec).

Efflux of thymidine from cells loaded with 5 mM thymidine into solutions of modified nucleosides at two different concentrations is summarized in Table 1. *Trans*-acceleration of thymidine outflow was observed in the presence of deoxyuridine, araU and araT, and efflux values equaled or exceeded those measured in the presence of the same concentrations of thymidine in the suspending medium. A slight acceleration of efflux into the 5 mM 3′-fluoro-3′-deoxy-araU solution was observed, but this did not occur in the presence of any of the other compounds at the concentrations tested.

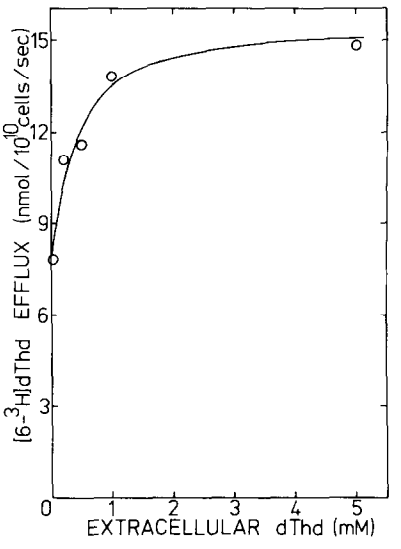


Fig. 2. *Trans*-acceleration of [6-^{3}H]thymidine efflux by extracellular thymidine at 25°. Cells were loaded with 5 mM [6-^{3}H]thymidine and suspended in buffered saline containing various concentrations of non-radioactive thymidine at a 5% hematocrit. Suspension in buffered saline alone resulted in an extracellular [6-^{3}H]thymidine concentration of 0.03 mM at zero time due to trapping of loaded permeant in the extracellular space. Transport was terminated by the addition of NBMPR. Each value represents the slope determined from at least four cell suspensions.

Efflux of thymidine into solutions of acyclothymidine was similar to that into buffered saline. On the other hand, 3′-chloro-3′-deoxy-araU, 3′-bromo-3′-deoxy-araU and acyclouridine moderately inhibited thymidine efflux at the highest concentrations tested, as efflux into solutions of these compounds was lower than that into buffered saline. Dideoxythymidine, 3′-iodo-3′-deoxy-araU and 3′-fluoro-3′deoxy-riboU all severely inhibited efflux of thymidine, so that it approached values near zero.

DISCUSSION

This investigation was undertaken to determine the effects of some structural modifications of pyrimidine nucleosides on their interaction with the mouse erythrocyte nucleoside transporter. The behavior of physiological nucleosides under the same conditions in this system was used as a basis for comparison. The experiments were carried out at 25°, a temperature which is convenient for measurement of the very rapid fluxes characteristic of nucleoside transport. Although transport kinetic parameters are expected to be temperature dependent, we have interpreted results of this work on the premise that transporter specificity will not be greatly affected by decreasing the temperature 12° below its physiological value.

Inhibition constants, determined from influx competition experiments, are assumed to reflect affinities for external binding sites on the transporter. *Trans*-acceleration of thymidine efflux from loaded cells into solutions containing nucleosides provides evidence that the extracellular nucleosides are trans-

located into the cell by the nucleoside transport mechanism. In terms of the simple carrier model, the phenomenon of *trans*-acceleration is compatible with a model in which the mobilities of loaded and empty carrier differ [33]; similarly, inhibition of efflux under these conditions may be interpreted as an inhibition of transporter re-orientation following release of outwardly transported thymidine. In the present work, combined results from studies of influx competition and *trans*-effects have provided a measure of the interaction of structurally modified nucleosides with the transporter.

Deoxyuridine, araU and araT were transported at rates comparable to that of thymidine, suggesting that a 2′-hydroxyl is not involved in the interaction of permant molecules with the nucleoside transport mechanism. This observation has been made previously [13] in studies using human erythrocytes; araU [34], araC [13, 35] and araA [14, 36, 37] are known to be permeants of the nucleoside transporter in various mammalian cells.

The present study has shown that modification at the 3′-position of pyrimidine nucleosides is not well tolerated by the transport mechanism. Replacement of the 3′-hydroxyl with either hydrogen or a halogen resulted in an increased K_i value, and, with the exception of 3′-fluoro-3′-deoxy-araU, inhibition of thymidine efflux. No evidence for translocation of these compounds across the cell membrane was obtained using *trans*-acceleration of efflux as a criterion, although these results do not exclude the possibility that these analogs utilize the carrier. Inhibition of thymidine efflux in the presence of these substances would be consistent with formation of a carrier–permeant complex with a mobility decreased in comparison with that of the empty carrier. The importance of a 3′-hydroxyl in the α-configuration in purine nucleoside uptake has been demonstrated by Taube and Berlin [15].

Of the five 3′-halogeno-3′-deoxynucleosides tested, 3′-fluoro-3′-deoxy-araU had the strongest affinity for the transporter, and it alone showed evidence of being translocated across the membrane. The contrasting behaviors of this compound and its ribo-isomer suggest that, while the configuration of the 2′-hydroxyl is not in itself of significance for transportability, substitution at the 3′-position may enhance the role of the configuration at the 2′-position in this process.

In view of the properties of certain acyclic "nucleosides" as anti-viral substances [38], or as inhibitors of uridine phosphorylase [7] and their possible use as modulators of pyrimidine nucleoside drugs *in vivo*, acyclothymidine and acyclouridine were included in the series of compounds investigated. These compounds represent an extreme modification of the sugar moiety as carbons 2′ and 3′ are absent. Their interaction with the transporter was slight, even at high concentrations, further emphasizing the importance of the 2′ and 3′-positions in transporter–permeant specificity. Our observations suggest that these compounds are poor substrates for the transporter and that possibilities for their passage across cell membranes may be limited to entry by a non-mediated process or by a different carrier mechanism, possibly a nucleobase transport mechanism.

The 2,2′-anhydro analog of araU is of particular interest because it lacks the conformational flexibility of the other compounds studied. Only a very weak interaction could be demonstrated in influx competition experiments, and no evidence for influx of the radiolabeled compound was obtained. The presence of a constrained furanose ring together with severely restricted rotation about the glycosidic bond in this compound may play a role in limiting its passage across the erythrocyte membrane.

In conclusion, tolerance of the nucleoside transporter to modifications in the sugar moiety of pyrimidine nucleosides is limited. Although the presence of individual substituents such as the 3′-hydroxyl appear to be vital features of permeant structure, it is likely that the combined effects of all sugar substituents in their determination of the nucleoside conformation are important in maximizing interaction with the transporter. Conformational flexibility may be a significant factor.

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