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Interaction of 2′-Halogeno-2′-deoxyuridines with the Human Erythrocyte Nucleoside Transport Mechanism

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SUMMARY

The efflux of radioactive thymidine from human erythrocytes at 25° was accelerated in the presence of extracellular 2′-fluoro-2′-deoxyuridine to a maximal velocity 120% of that observed in the presence of extracellular nonradioactive thymidine. Efflux in the presence of 2′-chloro-2′-deoxyuridine and 2′-bromo-2′-deoxyuridine did not exceed 56% and 49%, respectively. 2′-Iodo-2′-deoxyuridine did not accelerate thymidine efflux. In comparison, 2′-fluoro-2′-deoxycytidine and 2′-deoxyuridine accelerated thymidine efflux to maximal velocities of 170% and 91%, respectively. The half-saturation constant for acceleration of thymidine efflux by 2′-fluoro-2′-deoxycytidine was higher (0.90 mM) than those estimated for the other substances (0.22 mM or lower). Influx competition experiments at 25° showed that all of the above nucleosides competitively inhibited influx of thymidine into human erythrocytes. The K_m for the zero-*trans* influx of thymidine was 0.051 ± 0.008 mM, while the K_i values for 2′-deoxyuridine and the 2′-halogeno-2′-deoxyuridines were similar, ranging from 0.04 to 0.09 mM. The K_i for 2′-fluoro-2′-deoxycytidine was 0.18 mM. These results suggest that, although all nucleosides tested appeared to bind to the same transport site on the external membrane surface, their ease of transport through the membrane was determined by the properties of the halogen substituent at position 2′.

INTRODUCTION

Pyrimidine nucleosides having a halogen incorporated in the sugar moiety have received attention in recent years as potential diagnostics (1, 2) and as antitumor and/or antiviral substances (3). Although some metabolic studies have been carried out (4–7), little consideration has been given to rates of transport of these substances into mammalian cells, a factor which may determine their biological properties. In view of the fact that nucleosides are known to enter mammalian cells by a facilitated diffusion mechanism which is more sensitive to changes in the sugar moiety than in the nucleobase moiety (8), it is of particular interest to determine the effect of incorporation of halogen atoms in the sugar moiety on transportability.

This investigation was initiated to determine the transport properties of a group of 2′-halogeno-2′-deoxyuridines (Fig. 1) which were designed as diagnostic radiopharmaceuticals (1, 2). 2′-Deoxyuridine was included in the investigation for comparison, as was 2′-fluoro-2′-deoxycytidine, a cytotoxic nucleoside for which there is some evidence of transporter-mediated permeation in cultured cells (9). An attempt was made to determine whether the halogenated analogues enter mammalian cells by a mediated process and, if so, whether they are transported at rates comparable to those of the physiologically occurring nucleosides. We have used methods which allow evaluation of these substances as permeants in nonradioactive form. The nucleoside transport mechanism of human erythrocytes was chosen for the present study because it has a broad permeant specificity (8, 10, 11) and displays similarities to the transport systems of other mammalian cells (12, 13). It has been well characterized with respect to uridine transport (14–16) and shown to transport thymidine with equal facility (8). In addition, human erythrocytes do not metabolize uridine or thymidine (10) and so provide a system which does not complicate transport studies with the subsequent modification of permeants.

Two experimental approaches have been taken. In the first, the efflux of thymidine has been measured in the presence of extracellular nucleosides. Acceleration of the efflux of a known permeant by an extracellular substance (accelerative exchange diffusion) is evidence that the extracellular substance is transported through the membrane by a facilitated diffusion mechanism shared with the known permeant (17). In a second group of experiments, the analogues have been tested as inhibitors of

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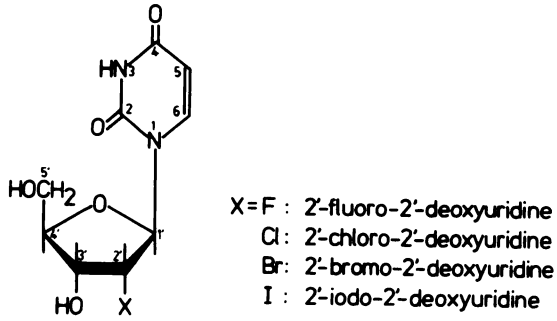


FIG. 1. *2′-Halogeno-2′-deoxyuridines*

thymidine influx under zero-*trans*[2] conditions. Competitive inhibition of nucleoside influx is evidence of interaction between inhibiting substances and a permeant binding site of the transport mechanism, although it does not demonstrate that the inhibiting substance utilizes the transport mechanism to move through the membrane.

EXPERIMENTAL PROCEDURES

Materials

Packed red blood cells from human blood which had been collected into anticoagulant citrate phosphate dextrose solution (USP) were obtained from the Red Cross Blood Transfusion Service (Edmonton, Alta.) and used 6–28 days after the collection date. [2-^{14}C]Thymidine was purchased from ICN Pharmaceuticals, Inc. (Irvine, Calif.) (55 mCi/mmole; radiochemical purity estimated by thin-layer chromatography to be 96%) and from Amersham Corporation (Oakville, Ont.) (58 mCi/mmole; radiochemical purity estimated by thin-layer chromatography to be 99%). 2′-Fluoro-2′-deoxyuridine, 2′-chloro-2′-deoxyuridine, 2′-bromo-2′-deoxyuridine, and 2′-iodo-2′-deoxyuridine were prepared using literature methods (1, 2). The compounds were estimated by thin-layer chromatography to be at least 95% pure. 2′-Fluoro-2′-deoxycytidine monohydrate (98%) was purchased from Calbiochem-Behring Corporation (La Jolla, Calif.). Thymidine (crystalline), 2′-deoxyuridine (98%), and *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid were purchased from Sigma Chemical Company (St. Louis, Mo.). NBMPR[3] was purchased from Terochem Laboratories Ltd. (Edmonton, Alta.). Aquasol-2 liquid scintillation fluid was purchased from New England Nuclear Corporation (Boston, Mass.). Other chemicals were reagent-grade from commercial sources.

Methods

All centrifugations were carried out in an Eppendorf 5412 microcentrifuge at 12,800 × *g*.

Accelerative exchange diffusion experiments. Efflux measurements were performed at 25° according to the procedure of Cass and Paterson (8), with minor modifications. Plasma and buffy coat were removed from the packed red cell fraction, and the erythrocytes were washed four times in buffered saline.[4] The cells were then suspended in a 10.0–10.5 mM solution of [2-^{14}C]thymidine (0.1 mCi/mmole) in buffered saline at a hematocrit of 40–50%, and incubated with mixing by inversion at 37° for 40 min to achieve an equilibrium distribution of thymidine inside and outside the cells. Cells were pelleted from the loading solution by centrifugation for 3 min; determination of radioactivity in the cell-free medium indicated that the cells were loaded to a concentration of 5.6–5.9 mM thymidine.

Each efflux time point was determined with a separate transport suspension as follows: 0.1-ml portions (containing approximately 10^9 cells) of packed, loaded cells were transferred[5] to a glass vial equipped with a magnetic stir bar. Buffered saline (1.0 ml) containing unlabeled nucleoside was added under magnetic stirring; after a specified time interval of 5, 10, or 15 sec, transport was stopped by the rapid addition of 1.0 ml of buffered saline containing 0.02 mM NBMPR, a specific inhibitor of nucleoside transport (18). After a further 10 sec, a 0.5-ml aliquot of the suspension was transferred to a 1.5-ml microcentrifuge tube and centrifuged for 1 min. Duplicate 0.1-ml aliquots of cell-free medium were immediately removed to scintillation vials. Immediate processing of samples in this way yielded efflux rates equivalent to those obtained in a separate experiment (data not shown) using centrifugation through dibutylphthalate. Zero-time values were determined by reversing the order of addition of NBMPR solution and nucleoside solution, using a 10-sec time interval. Aquasol-2 (10 ml) was added to each 0.1-ml aliquot of cell-free medium and the samples were counted by liquid scintillation procedures.

Influx competition experiments. Zero-*trans* influx of [2-^{14}C]thymidine was measured at 25° with and without inclusion of other nucleosides in the extracellular medium. Human erythrocytes were washed as described above and suspended in buffered saline at a hematocrit of 20%. Sixteen solutions of buffered saline containing thymidine at 0.026–0.22 mM and a test substance at 0–0.32 mM were prepared. The radioactive concentration in these nucleoside solutions was 2×10^6 cpm/ml. The procedure which follows was performed in duplicate for each of the 16 solutions. A 0.2-ml portion of the erythrocyte suspension was transferred to a 1.5-ml microcentrifuge tube, and at zero time, 0.2 ml of a nucleoside solution was rapidly added. After 3 sec, 0.4 ml of NBMPR stopping solution was jetted into the transport suspension from a 1-ml plastic syringe equipped with a plastic needle and thin polyethylene tubing. The suspension was immediately centrifuged for 1 min. Cell-free medium was removed by aspiration, 1.0 ml (except in the 2′-deoxyuridine experiment, where 0.5 ml was used) of stopping solution was added, and the cells were resuspended by brief vortex mixing. After recentrifugation and removal

[2] Zero-*trans* influx refers to transfer of nucleoside from the outside (*cis* side) of the membrane to the inside (*trans* side), where the concentration of nucleoside is assumed to be zero.

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

[4] Buffered saline contained 140 mM NaCl, 1.4 mM $MgSO_4$, and 18 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid, at pH 7.4.

[5] Reproducible dispensing of packed cells was achieved with the use of a solid interface micropipette (Socorex Isba S.A., Renens, Switzerland).

of cell-free medium, 0.5 ml of a 5% perchloric acid solution was added and the suspension was thoroughly mixed. The tubes were transferred to an ice bath and stored overnight at 4°. The mixtures were then centrifuged, and duplicate 0.15-ml aliquots of each supernatant were removed to scintillation vials, mixed with 10 ml of Aquasol-2, and counted.

Zero-time values were determined by first adding 0.2 ml of stopping solution to the cell suspension, and after 3 sec adding 0.4 ml of a solution containing equal volumes of the nucleoside solution and the stopping solution from the plastic syringe. Zero time values were independent of thymidine concentration. Three such values were determined in each experiment and their average radioactivity was subtracted from all other samples in determining the transport rates.

RESULTS AND DISCUSSION

Accelerative exchange diffusion experiments. Figure 2 shows examples of time courses of thymidine efflux in the presence of extracellular nucleosides. The plots were linear over the 15-sec time interval, indicating that the measured thymidine flux was unidirectional during this period. Zero time values indicated that thymidine trapped extracellularly in packed, loaded cells contributed, on the average, 0.03 mM thymidine to the extracellular medium in each suspension during efflux.

Efflux of thymidine in the presence of each concentration of test nucleoside was expressed as a percentage of thymidine efflux in the presence of 6 mM nonradioactive thymidine, determined for each batch of cells. This efflux accelerated by nonradioactive thymidine averaged 64 ± 8 pmoles/μl of packed cells per second (mean ± standard deviation of nine determinations representing erythrocytes from 5 units of blood). Efflux of thymidine into buffered saline without added nucleoside was about 36% of that measured in the presence of 6 mM thymidine (see Fig. 3).

Figure 3 shows how thymidine efflux varied with the concentration of extracellular nucleoside. For all substances tested except 2′-iodo-2′-deoxyuridine, thymidine efflux was greater in the presence of test substance than

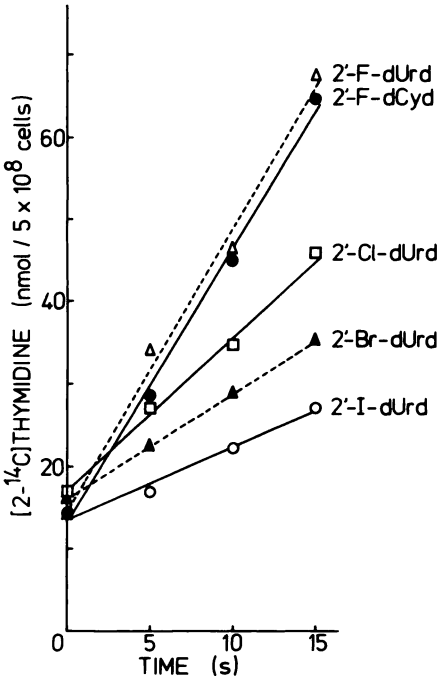


FIG. 2. *Examples of efflux measurements*

Experimental details are provided under Methods. Each point represents the measurement made with a separate transport suspension, or the mean value from two separate transport suspensions. The lines were fitted by the method of least squares. Slopes represent rates of thymidine efflux in the presence of extracellular 2′-fluoro-2′-deoxyuridine (△- - -△), 2′-fluoro-2′-deoxycytidine (●——●), 2′-chloro-2′-deoxyuridine (□——□), 2′-bromo-2′-deoxyuridine (▲- - -▲), and 2′-iodo-2′-deoxyuridine (○——○) at 1 mM concentrations.

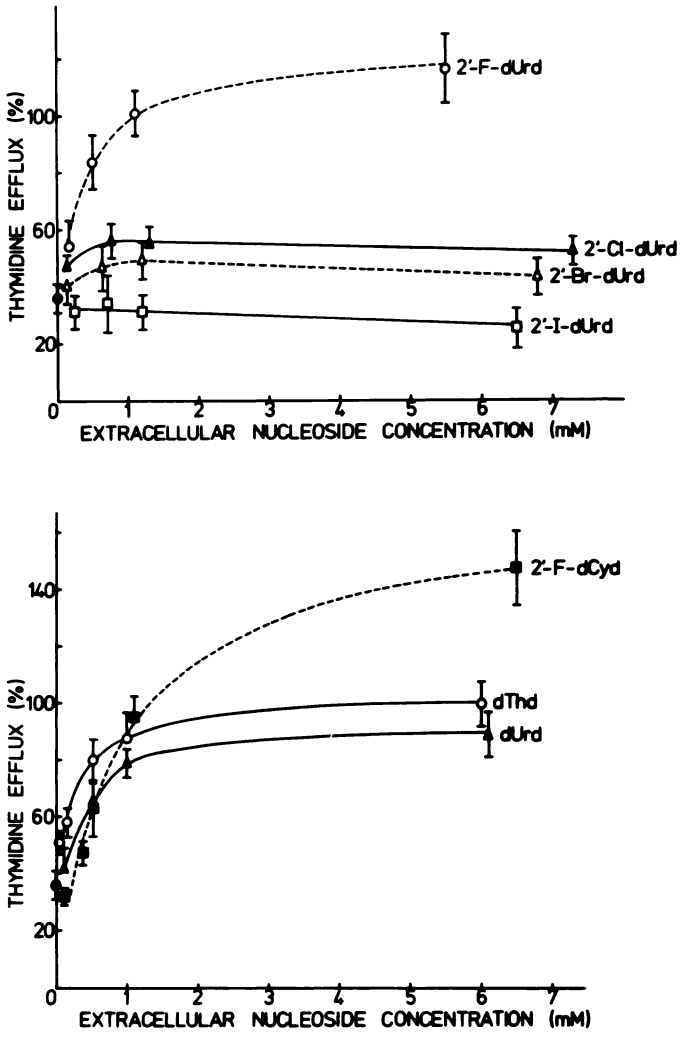


FIG. 3. *Dependence of thymidine efflux on extracellular nucleoside concentration*

Each point represents the slope of an efflux curve (examples in Fig. 2) expressed as a percentage of thymidine efflux in the presence of 6 mM extracellular thymidine. Vertical lines indicate the standard deviation of each value. The extracellular nucleoside concentrations shown do not include the 0.03 mM thymidine contribution from residual thymidine trapped in packed, loaded cells (see Results and Discussion). Extracellular nucleosides tested were: (*top*) 2′-fluoro-2′-deoxyuridine (○- - -○), 2′-chloro-2′-deoxyuridine (▲——▲), 2′-bromo-2′-deoxyuridine (△- - -△), and 2′-iodo-2′-deoxyuridine (□——□), and (*below*) 2′-fluoro-2′-deoxycytidine (■- - -■), thymidine (○——○), and 2′-deoxyuridine (▲——▲). The single *solid circle* and its standard deviation shown on the ordinate of each graph indicates the value for thymidine efflux in the absence of added extracellular nucleoside, determined by adding buffered saline to the loaded cells.

in buffered saline alone, and it increased with the concentration of extracellular nucleoside. This demonstration of accelerative exchange diffusion is evidence that the substances tested, except 2′-iodo-2′-deoxyuridine, share the thymidine transport mechanism.

The data shown in Fig. 3 were used without subtraction of the value for efflux in the absence of added extracellular nucleoside, to estimate kinetic parameters for this experimental system according to Hanes (19). Plots of S/v versus S for the acceleration of thymidine efflux in the presence of 2′-deoxyuridine, 2′-fluoro-2′-deoxyuridine, 2′-fluoro-2′-deoxycytidine, and thymidine are shown in Fig. 4. The linearity of these plots indicates that efflux measured in the presence of these substances was saturable.

K_m and V_{max} values derived from accelerative exchange diffusion experiments are influenced by the ratio of the rates of movement of loaded and unloaded carrier (17) and are not easily related to values determined under other experimental conditions. Kinetic parameters for uridine transport in human erythrocytes measured under equilibrium exchange conditions have been shown to differ from those determined in zero-*trans* experiments (15). In the present study, no attempt has been made to relate half-saturation constants from accelerative exchange diffusion experiments to K_i values obtained in the influx competition experiments (below), but kinetic parameters have been determined for accelerative exchange diffusion in order to compare the behavior of the 2′-halogenated analogues with that of thymidine and 2′-deoxyuridine in this experimental system. The parameters are referred to as simply the maximal velocity and the half-saturation constant, which is the concentration of extracellular nucleoside at which thymidine efflux was half-maximal. It is assumed that the half-saturation constants indicate the relative affinities of the substances for the transport mechanism under these experimental conditions.

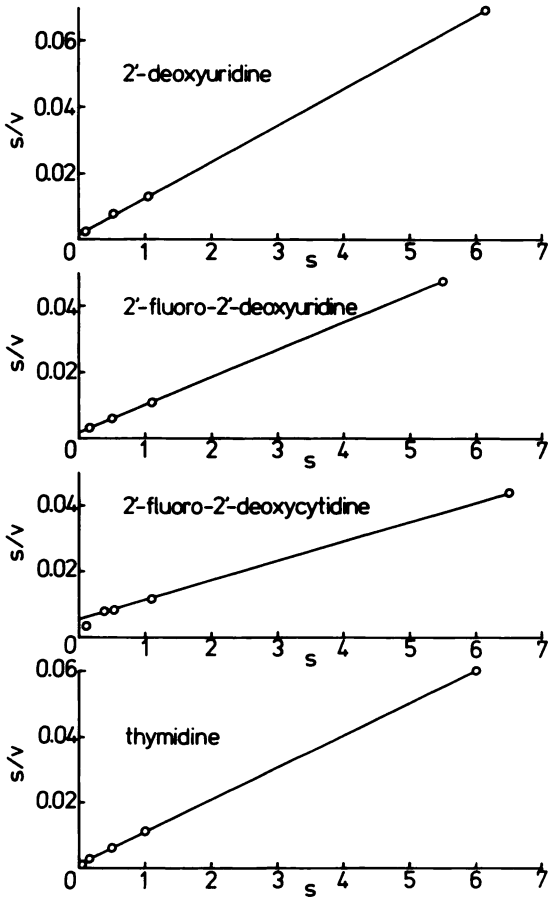


FIG. 4. *Estimation of kinetic parameters for the acceleration of thymidine efflux*

Data from Fig. 3 were plotted according to Hanes (19). The ordinate shows extracellular nucleoside concentration (millimolar) · [efflux (%)]$^{-1}$, while the abscissa shows extracellular nucleoside concentration (millimolar). The lines were fitted by the method of least squares. Kinetic parameters are summarized in Table 1.

The kinetic parameters for 2′-deoxyuridine, 2′-fluoro-2′-deoxyuridine, 2′-fluoro-2′-deoxycytidine, and thymidine are summarized in Table 1. The highest maximal velocities were those estimated for 2′-fluoro-2′-deoxycytidine and 2′-fluoro-2′-deoxyuridine, which exceeded that of its parent compound, 2′-deoxyuridine. Maximal velocities for thymidine and 2′-deoxyuridine accelerated efflux of thymidine are comparable to those obtained by Cass and Paterson (8) from measurements of uridine efflux from human erythrocytes. The half-saturation constant for 2′-fluoro-2′-deoxycytidine was higher than those estimated for the other substances, suggesting that its affinity for the transporter is lower.

Data for 2′-chloro-2′-deoxyuridine, 2′-bromo-2′-deoxyuridine, and 2′-iodo-2′-deoxyuridine did not allow a precise determination of kinetic parameters, although visual examination of the Fig. 3 plots suggests that their half-saturation constants would lie below 0.2 mM. Thymidine efflux in the presence of 2′-chloro-2′-deoxyuridine and 2′-bromo-2′-deoxyuridine did not exceed 56% and 49%, respectively.

The presence of a bulky substituent on the sugar moiety of pyrimidine nucleosides has been shown (8) to reduce greatly their ability to accelerate the efflux of uridine from human erythrocytes. In the present investigation, substitution with a series of halogens at position 2′ of 2′-deoxyuridine brought about a decrease in ability to accelerate thymidine efflux which paralleled the increase in size and/or decrease in electronegativity of the halogen atoms (20).

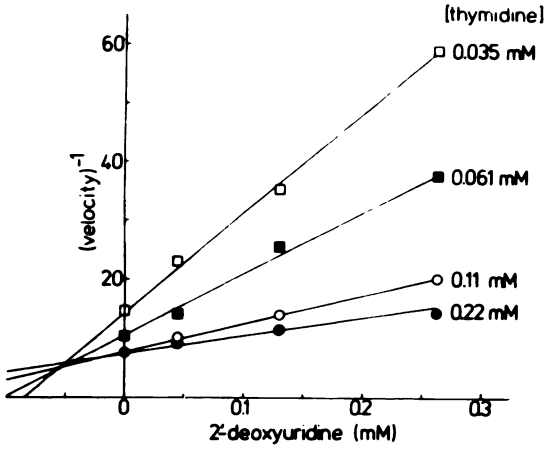
Influx competition experiments. Influx measurements over 3-sec time intervals were assumed to represent initial rates. This assumption is consistent with the data of Fig. 2 and is supported by results of a thymidine influx experiment (data not shown) which demonstrated that

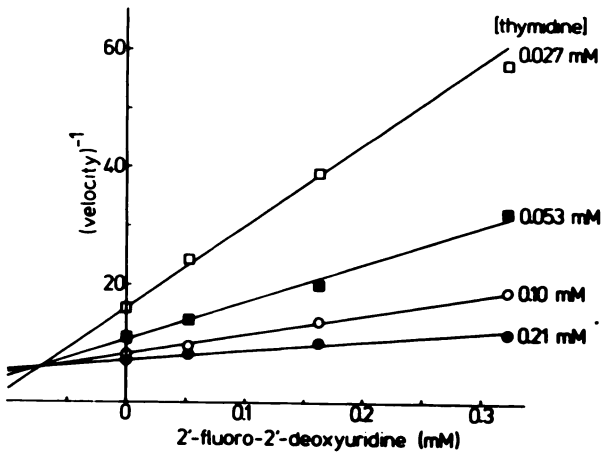
TABLE 1

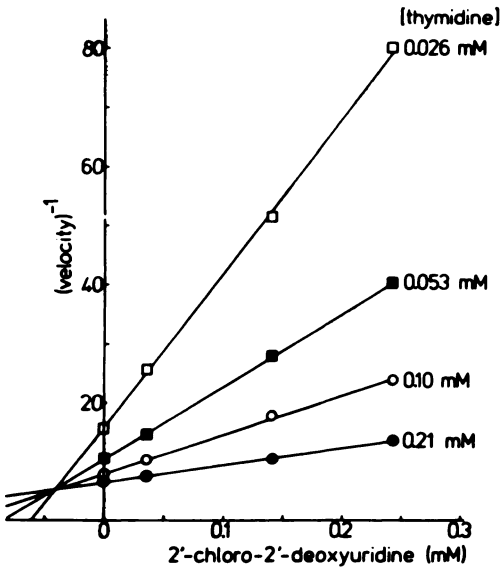
Kinetic parameters estimated from accelerative exchange diffusion experiments

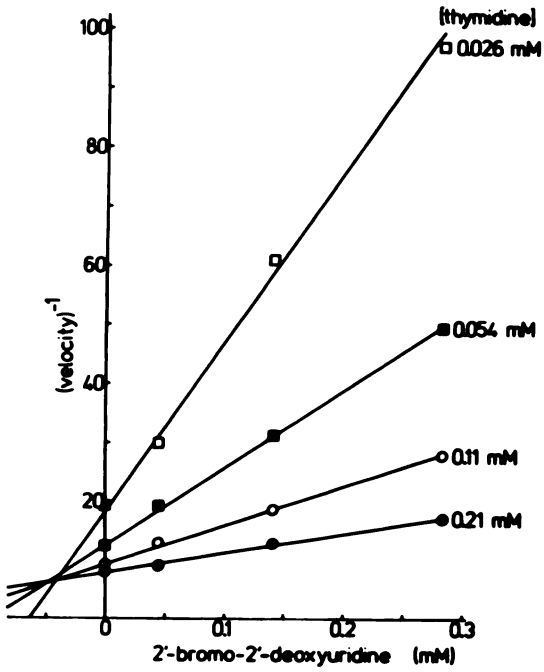
Extracellular nucleoside	Maximal velocity[a]	Half-saturation constant ± SD
	%	mM
2′-Deoxyuridine	91	0.16 ± 0.03
2′-Fluoro-2′-deoxyuridine	120	0.22 ± 0.01
2′-Fluoro-2′-deoxycytidine	170	0.90 ± 0.05
Thymidine	100	0.11 ± 0.02

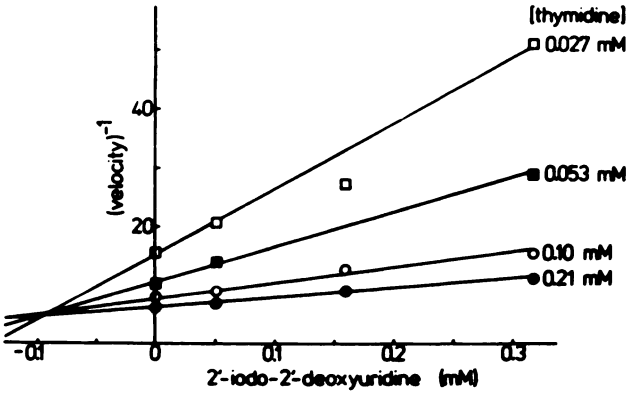
[a] Expressed as a percentage of [2-^{14}C]thymidine efflux in the presence of 6 mM nonradioactive thymidine.











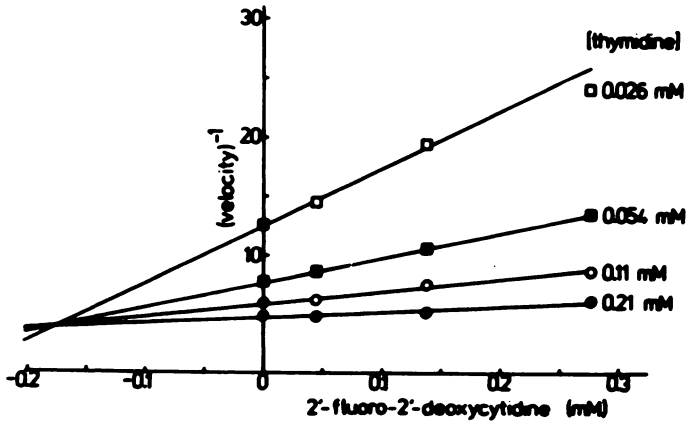


FIG. 5. *Estimation of inhibitor constants according to the method of Dixon (21)*

Plotted values are the means of duplicate aliquots of the perchloric acid extract from a single transport suspension. Experimental details are provided under Methods. Zero-time radioactivity was, on the average, about 11% of 3-sec influx radioactivity and was subtracted from the 3-sec values in determining initial velocities. The ordinate has units of (pmoles/10 μl of packed cells/hr)$^{-1}$. Each line is labeled with the thymidine concentration used. The estimated K_i values are summarized in Table 2.

the 3-sec interval was part of a linear time course in the influx of 0.1 mM thymidine. These initial rates were used to estimate inhibitor constants by plotting $(\text{velocity})^{-1}$ versus $[i]$ according to the method of Dixon (21). Figure 5 shows the results of these studies. In all cases, the straight lines intersected at a point above the $[i]$ axis, indicating either competitive or mixed inhibition. Plots of the data according to Cornish-Bowden (22) excluded the possibility that mixed inhibition was responsible for these results. Thus, all substances tested appeared to compete with thymidine for the same binding site.

K_m and V_{max} values for the zero-*trans* influx of thymidine were estimated from data from these experiments in which thymidine influx was measured in the absence of competing permeant. Plotting the data according to Hanes (19) (data not shown) yielded $K_m = 0.051 \pm 0.008$ mM and $V_{max} = 5.3 \pm 1.0$ pmoles/μl of packed cells per second (mean values ± standard deviation of six determinations). The K_m is similar to values reported for the influx of thymidine into various cultured mammalian cells (23), and comparable to the K_m value of 0.073 ± 0.069 mM for the zero-*trans* influx of uridine into human erythrocytes, as determined by Cabantchik and Ginsburg (15).

Table 2 summarizes the K_i values estimated from Dixon plots. K_i values for the 2′-halogeno-2′-deoxyuridines were similar to that for 2′-deoxyuridine and to the K_m for thymidine. Assuming that these constants are measures of affinity, these results suggest that all of the above substances bind well to the transporter. On the other hand, the K_i for 2′-fluoro-2′-deoxycytidine was somewhat greater. This is consistent with the fact that the half-saturation constant determined in accelerative exchange diffusion experiments was higher for this substance than for the other nucleosides.

Correlation of the behavior of the halogenated analogues in the two experimental systems. The present investigation has shown that while all of the 2′-halogenated pyrimidine nucleoside analogues tested competitively inhibited thymidine influx into human erythrocytes, their acceleration of thymidine efflux varied in a way which could be related to the properties of the halogen substituent. According to Stein (17), accelerative exchange diffusion is possible only in systems in which loaded carrier moves[6] through the membrane more rapidly than unloaded carrier. Experimental data consistent with the conclusion that the rates of movement of loaded and unloaded carrier can differ have been provided by Cabantchik and Ginsburg (15), who infer that movement of the unloaded carrier is the rate-limiting step in the transport of uridine in human erythrocytes. We suggest from the present results that the rate of movement of the loaded carrier is affected by the properties of a substituent at position 2′ of the nucleoside with which the carrier is complexed. This would allow transport velocities to differ among a group of compounds whose affinities for the carrier are similar. In this work, while the affinities of 2′-chloro-2′-deoxyuridine, 2′-bromo-2′-deoxyuridine, and 2′-iodo-2′-deoxyuridine at the external face of the transporter were similar to those of thymidine and 2′-deoxyuridine, their abilities to utilize the transporter to move through the membrane were hindered to varying degrees.

[6] We interpret this term to include translocation and/or conformational change.

TABLE 2

Inhibitor constants estimated from influx competition experiments

Kinetic constants obtained in the absence of competing substrate for the zero-*trans* influx of thymidine were $K_m = 0.051 \pm 0.008$ mM and $V_{max} = 5.3 \pm 1.0$ pmoles/μl of packed cells per second (values are means ± standard deviation from six experiments).

Nucleoside	K_i
	mM
2′-Deoxyuridine	0.05
2′-Fluoro-2′-deoxyuridine	0.07
2′-Chloro-2′-deoxyuridine	0.04
2′-Bromo-2′-deoxyuridine	0.04
2′-Iodo-2′-deoxyuridine	0.09
2′-Fluoro-2′-deoxycytidine	0.18

Although we have been unable in these studies to provide evidence for the mediated transport of 2′-iodo-2′-deoxyuridine, our results do not exclude the possibility that this analogue utilizes the carrier. It is conceivable that the rate of movement of the carrier when loaded with this substance does not exceed the rate of movement of the unloaded form. Such a situation would be consistent with our observations. In any case, 2′-chloro-2′-deoxyuridine, 2′-bromo-2′-deoxyuridine, and 2′-iodo-2′-deoxyuridine appear to be less suitable as permeants of this transport mechanism than are thymidine, 2′-deoxyuridine, and the 2′-fluoro-analogues tested.

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