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TRANSPORT OF PYRIMIDINE NUCLEOSIDES ACROSS HUMAN ERYTHROCYTE MEMBRANES*

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SUMMARY

The transport of pyrimidine nucleosides in human erythrocytes has been shown to occur by a facilitated diffusion mechanism. Transport studies of these nucleosides are not complicated by metabolic conversions as in the case of the action of phosphorylases and deaminases on purine nucleosides. The transport carrier K_m and v_{max} were K_m = 3.5, 1.7, 1.3 and 0.7 mM and v_{max} = 16.5, 6.9, 4.7 and 10.0 mM/min for cytidine, cytosine-β-arabinoside, thymidine and uridine, respectively. The initial rates for nucleoside exit competition experiments are in good agreement with those calculated using the above K_m's and v_{max}'s. Some preliminary evidence that the pyrimidine and purine nucleosides are transported by a common system is also presented.

INTRODUCTION

The transport of purine bases and of purine nucleosides into human erythrocytes has been studied by a number of investigators[1-6]. In the case of free purine bases, facilitated diffusion appears to be the chief mechanism of translocation. There are indications that a second, more complex mechanism is operative at high purine concentration[4]. Similar statements concerning the modes of transport of purine of pyrimidine nucleosides could not be made at the outset of this research. In addition, the permeability of pyrimidine nucleosides to the human erythrocyte membrane was not clearly established. Estimation of the kinetic parameters for purine nucleosides from LOWY'S[6] or WHITTAM'S[5] data on their partial uptake by erythrocytes was not possible. Furthermore, the transport of purine nucleosides has been shown to be complicated by the action of purine nucleoside phosphorylase and 6-amino purine deaminase, making more deliberate attempts at kinetic measurements of uptake difficult and exit measurements impossible to conduct[6].

The purpose of this study was to obtain accurate kinetic measurements for the translocation of pyrimidine nucleosides, to establish their mechanism of transport and their relationship to purine nucleoside transport. The existence and the relationships between specific transport systems for different types of sugars and sugar derivatives

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is a matter of continuing interest. The ribosides of uracil, cytosine, the deoxy riboside of thymine and the arabinoside of cytosine were studied. They all appear to enter the cell by a facilitated diffusion mechanism and in contrast with purine nucleosides, are metabolically inert in human erythrocytes during the time required to perform the kinetic measurements reported herein. Preliminary information on the relationships which purine nucleosides hold to the transport of pyrimidine nucleosides will also be reported.

MATERIALS AND METHODS

Human erythrocytes (Type A, Rh^+), collected from acid–citrate–dextrose blood, were obtained from the Southwest Michigan Blood Bank and stored under sterile conditions at 4°. Cells were used within 21 days of the bleeding. Before use, the cells were washed a minimum of 3 times with buffered saline solution (0.145 M NaCl, 0.01 M phosphate, pH 7.4). The $HgCl_2$ stopping solution contained $HgCl_2$ (2.0 mM), KI (1.25 mM) and NaCl (0.34 M). Scintillation fluid was prepared from *p*-bis-2′-(5-phenyloxazoyl)benzene (0.2 g), 2.5-diphenyloxazole (8.0 g), ethylene glycol monoethyl ether (1.0 l) and toluene (1.0 l). The radiochemicals [^{14}C]inosine, [2-^{14}C]uridine, [^{3}H]cytidine, methyl-[^{3}H]thymidine, and [5-^{3}H]cytosine-β-arabinoside were all obtained from Amersham/Searle.

Metabolic stability of pyrimidine nucleosides

A suspension containing 2 ml of washed cells and 4.5 ml of the labeled pyrimidine nucleoside was incubated for 16 h at 37°. After separation by centrifugation, the cells were lysed with water (5.0 ml) and the protein precipitated with an equal volume of 10 % trichloroacetic acid. Aliquots from the supernatant before and after cell lysis were examined by thin-layer chromatography on silica gel previously equilibrated for 1 h with ammonia–methanol–chloroform (1:2:2, by vol.). Chromatograms were developed with the same solvent conditions. The spots were detected by ultraviolet light and the portions of the chromatogram from the top of the nucleoside spot to the solvent front and from the origin to the spot were examined as well as the spot. The silica gel was scraped into a vial of scintillation fluid and counted. The distribution of counts in each chromatogram was noted.

Inhibition of pyrimidine nucleoside transport by Hg^{2+}

A 10% suspension of the erythrocytes was incubated for 4 h with a solution which was 40 mM in labeled nucleoside. All four pyrimidine nucleosides were examined. A portion of the solution was adjusted to a hematocrit of 50 % by centrifugation and removal of supernatant. After lysis of the cells, precipitation and centrifugation of the protein by the procedure given above, the supernatant was counted. Correcting for counts from the incubation medium, the concentrations of the nucleosides in the erythrocytes were calculated.

To six separate 100-μl samples of the 50 % suspension 5 ml of the ice cold $HgCl_2$ stopping solution was added while stirring. One sample was centrifuged immediately at 2000 $\times$ *g* and the supernatant removed. This procedure required 50 sec. The remaining 5 samples were centrifuged after stirring in the cold for 1,2, 3, 4 and 5 min. This procedure was repeated using ice cold isotonic saline in place of the $HgCl_2$

stopping solution. The cells in these centrifuged fractions were lysed and counted as above. A representative nucleoside, thymidine, was similarly examined at 37°. Samples were taken at 0,1,2 and 3 min.

Uptake kinetic experiments

Uptake of labeled pyrimidines was followed from prepared solutions of the radiochemical in 10% suspensions of the red cells at 37°. At appropriate times 500-μl aliquots were added to 5.0 ml of ice cold $HgCl_2$ stopping solution. The samples were immediately centrifuged at 2000 $\times$ *g* for 50 sec. After removal of the supernatant by suction, 0.5 ml of water was added to lyse the cells. The protein was subsequently precipitated by addition of 0.5 ml of 10% trichloroacetic acid. After centrifugation for 5 min, 400 μl of the supernatant was added to 10 ml of scintillation fluid and counted in a Packard Tricarb spectrometer.

In uptake competition experiments 50 μl of packed cells were added to 450 μl of a mixture of unlabeled inhibitor (20 mM) and labeled pyrimidine nucleoside (10 mM). Aliquots were taken at various times and treated as in simple uptake experiments.

Exit kinetic experiments

To follow the exit of labeled nucleoside a 40% suspension of cells was first preloaded for 4 h at 37° with constant stirring. The suspension was centrifuged and adjusted to a hematocrit of 50% by removal of supernatant. Aliquots of 100 μl were added to 2.0 ml of buffered saline at 37°. The efflux was terminated by adding 5 ml (10 ml in some experiments) of the ice cold $HgCl_2$ stopping solution. These cell suspensions were subsequently treated as in the uptake experiments. From 12–14 time points were determined in a typical kinetic run.

In exit competition experiments 2 ml of packed cells were incubated for 4 h at 37° with 5 ml of labeled pyrimidine nucleoside together with the unlabeled competing species. After the incubation a portion of the cell suspension was centrifuged and the internal concentration of the labeled nucleoside was determined as in the uptake experiments. For most runs, separate mixtures containing labeled competing species and the unlabeled test nucleoside were used in parallel incubations to determine the internal concentration of the competing species. Concentrations of the test nucleoside varied between 4.5 and 7.0 mM. The cell suspension was adjusted to a hematocrit of 50% by centrifugation and removal of supernatant at 37°. Aliquots of 100 μl were added to 2.0 ml of buffered saline containing the same concentration of the unlabeled competing species as was found inside the cells. The internal concentration of test nucleoside was determined at a number of times as in the uptake experiments.

Determination of K_m and v_{max}

For a given nucleoside a number of exit kinetic plots were determined starting from the same internal concentration in each case. Several different concentrations of labeled nucleoside in buffered saline were used as the exiting media. Initial internal concentrations varied between 9 and 35 mM while external concentrations between 1 and 6 mM were usually employed. K_m and v_{max} were calculated for each nucleoside from these exit kinetic plots as described in calculations.

Calculations

The method of SEN AND WIDDAS[7] was used to determine the kinetic parameters K_m and v_{max}. The symbols employed by STEIN[8] are used here. It is assumed that the system is described by Eqn. 1 where S_i is the concentration of nucleoside inside the cells at time t.

$$\frac{dS_i}{dt} = \left(\frac{S_e}{S_e + K_m} - \frac{S_i}{S_i + K_m}\right) v_{max} \quad (1)$$

K_m is the nucleoside concentration at which the flux is one half the limiting flux, v_{max}. In the experiments the exit of nucleoside was followed from cells preloaded at concentration S_0 into various external nucleoside concentrations, S_e. The external volume was 60-fold larger than the internal volume. Thus S_e was assumed to remain constant during the experiment. For each external nucleoside concentration a plot of S_i against t was made and the exit time, t_e, determined by the intercept on the $S_i = S_e$ line of a line drawn tangent to the curve at $t_0 = 0$.

In general

$$t_e = \frac{(S_0 + K_m)(S_e + K_m)}{K_m v_{max}} \quad (2)$$

When S_e varies while S_0 remains constant, a plot of t_e against S_e yields a straight line with the intercept on the S_e axis equal to $-K_m$ and the intercept on the t_e axis equal to $(S_0 + K_m)/v_{max}$. K_m and v_{max} were determined by a least squares fit.

If the concentration of the competing nucleoside is designated by S_e in Eqn. 1 and its half saturation constant by K_i, then substitution of the expression $K_m(1 + 1/K)_i$ for K_m in Eqn. 1 allows calculation of the initial velocities due to competitive inhibition of the transport of the test nucleoside. In order to describe the entire course of a simple or competition exit kinetic plot, S_i was expressed as a function of t in the integrated form of Eqn. 1 where S_e is assumed to be constant (Eqn. 3).

$$K_m v_{max} t = (S_e + K_m)(S_0 - S_i) + (S_e + K_m)^2 \ln \frac{S_e - S_0}{S_e - S_i} \quad (3)$$

RESULTS

Metabolic stability of pyrimidine nucleosides

Each of the four pyrimidine nucleosides, incubated in red cell suspensions, was examined by thin-layer chromatography and the results compared with the corresponding untreated nucleoside. Both intracellular and extracellular fractions were examined. All nucleoside fractions gave single spots. The R_F values for the treated and untreated nucleosides were indentical. All of the counts applied were recovered in the eluted spots. Representative data for the supernatant and cell lysate fractions are shown for the four nucleosides in Table I.

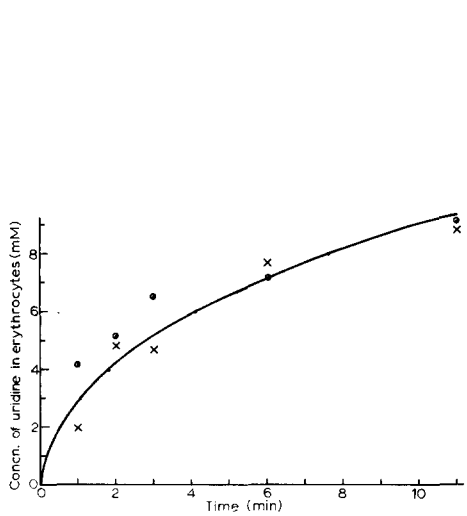
Two additional experiments also indicate the lack of metabolic transformation. First, packed cells were incubated with an equal volume of 80 mM uridine for 5 h at 37°. The supernatant was diluted to 20 mM and uptake experiments were done comparing this material to a freshly prepared solution of 20 mM uridine. The uptake in the two cases is shown in Fig. 1. Second, the internal concentration of nucleoside calculat-

TABLE I

DISTRIBUTION OF RADIOACTIVITY (counts/min) ELUTED FROM SILICA GEL AFTER THIN-LAYER CHROMATOGRAPHY OF INCUBATION FRACTIONS OF PYRIMIDINE NUCLEOSIDES WITH HUMAN ERYTHROCYTES

Zone 1, 2 cm strip from 1 cm below origin to 1 cm below center of spot; Zone 2, 2 cm × 2 cm square centered about nucleoside spot; Zone 3, 2 cm strip from 1 cm above center of the nucleoside spot to the solvent front.

	Supernatant fraction			*Cell lysate fraction*		
	Zone 1 *Origin to spot*	*Zone 2* *Nucleoside spot*	*Zone 3* *Spot to front*	*Zone 1* *Origin to spot*	*Zone 2* *Nucleoside spot*	*Zone 3* *Spot to front*
Cytidine	3	474	0	12	123	0
Cytosine-β-arabinoside	14	540	7	5	174	0
Thymidine	0	960	4	0	223	0
Uridine	16	656	30	7	142	2



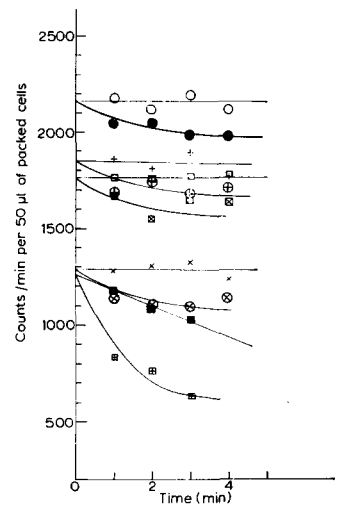


Fig. 1. Uptake of uridine and uridine which had been incubated with erythrocytes. The uptake of radioactivity by erythrocytes from 20 mM solutions of [^{3}H]uridine (⊙) and [^{3}H]uridine (×) from the recovered supernatant fraction after incubation with erythrocytes for 16 h at 37°. The curve was calculated from the integrated rate equation (Eqn. 3) using the values of K_m and v_{max} given in Table II.

Fig. 2. Exit of pyrimidine nucleosides from erythrocytes into $HgCl_2$ and isotonic buffer. The counts remaining in 50 µl of cells previously equilibrated with labeled nucleoside (40 mM) are plotted against the times nucleosides were allowed to exit into isotonic buffer or $HgCl_2$ stopping solution. Parallel experiments at 0–5° are shown for cytosine-β-arabinoside (○, ●), thymidine (+, ⊕), cytidine (□, ⊠), and uridine (×, ⊗) for $HgCl_2$ and isotonic buffer solution exits respectively. The exit of thymidine (31 mM) from erythrocytes at 37° into isotonic buffer (squares with vertical cross) and into $HgCl_2$ solution (■) is also shown.

ed on a cell water basis has never been found to be greater than the external concentration. Experiments with inosine indicate that such metabolites are easily formed in purine nucleoside systems. When a 20% suspension of erythrocytes was incubated for 16 h at 37° with 10 mM [^{14}C]inosine 5.2 times as many counts as the expected

equilibrium value were found in the cells. This was taken as evidence of extensive conversion of inosine to nontransportable materials, *e.g.* IMP, ITP, *etc.*[9]. After equilibration with 40 vol. of buffer for 1 h at 37°, 47 % of the radioactivity remained within the cells. In a separate experiment fresh cells took up only 12 % as many counts from the radioactive material from this intracellular fraction as were taken up from a solution of untreated nucleoside of the same apparent concentration.

Termination of pyrimidine nucleoside transport

Efflux from cells preequilibrated with each of the four pyrimidine nucleosides was examined as a function of added $HgCl_2$ stopping solution or isotonic saline at 0–5° in one set of experiments and 37° in another. Experiments at 37° were conducted with thymidine only. The results are shown in Fig. 2. After mixing each of the added solutions with each of the cell suspensions and rapidly centrifuging, the cells were analyzed for nucleoside content. Parallel analyses were conducted at 0–5° after mixing of cell suspensions with added solutions for 1,2,3,4 and 5 min. Similarly, experiments at 37° were conducted for 1,2 and 3 min. The total counts isolated under parallel conditions for the experiments at 0–5° for times up to 5 min fall only slightly (5–10 %) beyond the range of experimental precision of the analysis (5 %). At 37° cells preequilibrated with thymidine (31 mM) were observed to retain 90 % of their counts after 1 min exit into the $HgCl_2$ stopping solution whereas only 55 % of the counts remained when cells were allowed to exit for the same time into isotonic saline. The results indicate the addition of ice-cold $HgCl_2$ solutions offers a satisfactory

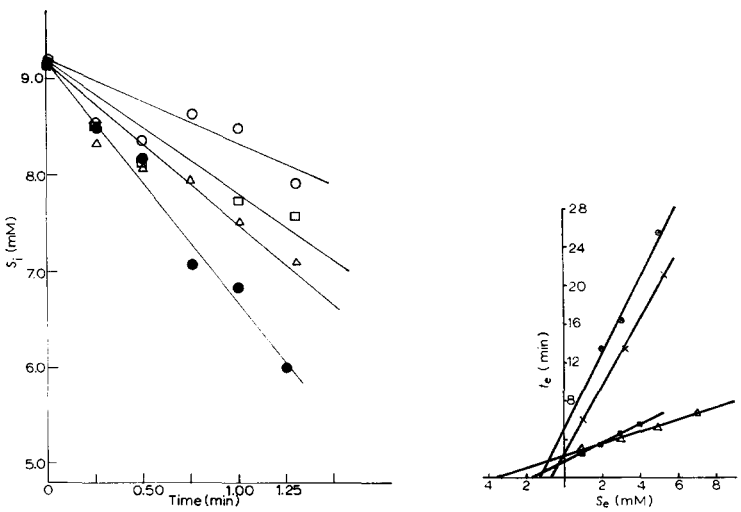


Fig. 3. Exit of cytosine-β-arabinoside into buffer containing various concentrations of cytosine-β-arabinoside. The internal concentration of [5-^{3}H]cytosine-β-arabinoside is plotted as a function of time of exit into buffered saline solutions containing 1.0, 2.0, 3.0 and 4.0 mM [5-^{3}H]cytosine-β-arabinoside (●, △, □ and ○, respectively).

Fig. 4. Determination of K_m and v_{max} for various nucleosides. The exit times, t_e, as determined from plots such as the one shown in Fig. 3 are plotted against the corresponding initial external concentrations of permeant, S_e, for cytidine (△), cytosine-β-arabinoside (■), thymidine (⊙), and uridine (×). The lines were fit by least squares.

method of terminating transport and measuring the concentration of nucleoside within the cells.

Determination of K_m and v_{max}

The initial rates of exit at constant S_0 for a number of S_e's for the four nucleosides studied were used to estimate t_e's. Fig. 3 illustrates exit plots for cytosine-β-arabinoside. T_e's, obtained from these and similar data, were plotted against S_e as shown in Fig. 4. The intercepts determined from the least squares fit of the points were used to calculate the K_m and v_{max} for each of the nucleosides as indicated under calculations. The values of K_m and v_{max} are given in Table II.

Exit competition experiments

In these experiments equal concentrations of the competing nucleoside were maintained internally and externally. All pyrimidine nucleosides studied here were

TABLE II

VALUES OF K_m AND v_{max} FOR VARIOUS PYRIMIDINE NUCLEOSIDES

	S_0 (*mM*)	K_m (*mM*)	v_{max} (*mM/min*)
Cytidine	33.6	3.5	16.5
Cytosine-β-arabinoside	9.2	1.7	6.9
Thymidine	24.2	1.3	4.7
Uridine	25.5	0.7	10.0

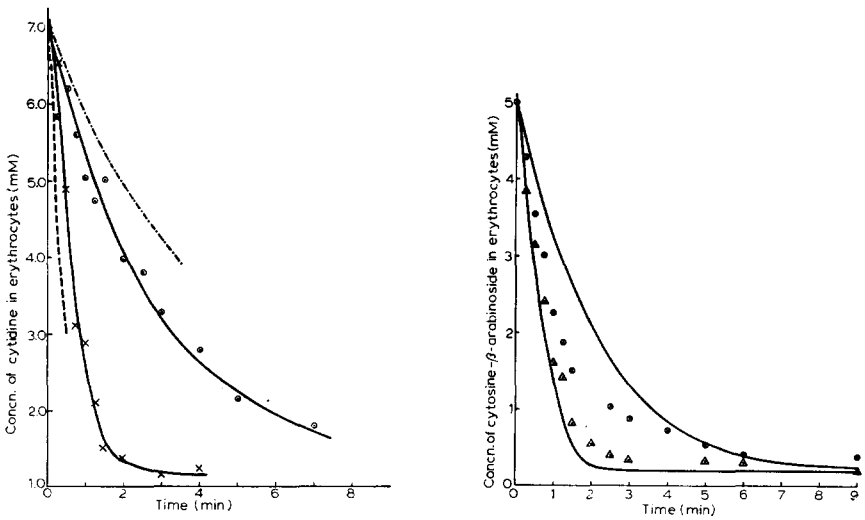


Fig. 5. Influence of uridine on the exit of cytidine from erythrocytes. The internal concentration of [^{14}C]cytidine is plotted as a function of time in the absence (×) and presence of 20 mM uridine (⊙). The calculated plots for the simple (---), and competition (–·–) exits are shown.

Fig. 6. Effect of cytidine on the efflux of cytosine-β-arabinoside from erythrocytes. The internal concentration of [5-^{3}H]cytosine-β-arabinoside is plotted against time in the absence (△) and presence of 20 mM cytidine (⊙). The time course for simple and competition exit was calculated from of Eqn. 3.

demonstrated to compete with at least one other member of the series. The exit of cytidine in the presence and absence of uridine is shown in Fig. 5. The exit rate of cytidine is greatly diminished in the presence of uridine. The exit of cytosine-β-arabinoside in the presence and absence of cytidine is shown in Fig. 6. The exit rate of cytosine-β-arabinoside shows a small reduction when in competition with cytidine.

The theoretical exit curves calculated from Eqn. 3 are shown in Fig. 5 and Fig. 6. Analysis of the experimental precision of K_m determinations suggests observed differences between the experimental and calculated plots is not critical.

Relationships between purine and pyrimidine nucleoside transport

The well documented metabolic interconversion of purine nucleosides occurring in erythrocytes makes exit experiments from preloaded cells less attractive than uptake competition studies. In the latter there is less chance for transformation of purine nucleosides to occur. The results of an uptake competition study are shown in Fig. 7. In these studies no attempt could be made to maintain inhibition concentrations at constant values as in the exit competition experiments. Only major changes in the apparent initial velocities of uptake were taken as meaningful. It is clear that the uptake inhibition of thymidine by uridine is detectable in this experiment and that both sucrose and inosine inhibit uridine uptake.

Further evidence bearing on a possible relationship between purine and pyrimidine nucleoside transport was observed from a study of the inhibition of uptake of thymidine and inosine by cells pretreated with fluorodinitrobenzene. Incubation of a solution of 10 % packed cells with, 2.5 mM fluorodinitrobenzene at 25° for 2 h gave cells which took up 26 % and 30 % as much thymidine and inosine as the respective controls.

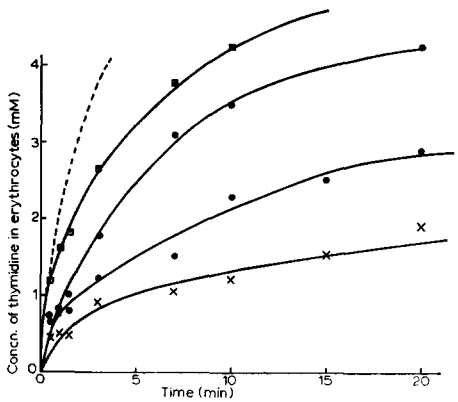


Fig. 7. The uptake of thymidine in the presence of different substances. The uptake of radiolabeled thymidine is plotted against time in the presence of 20 mM concentrations of purine (⊡), sucrose (●), uridine (⊙), and inosine (×). The calculated initial uptake curve based on data from Table II is plotted for thymidine (---).

DISCUSSION

The measurements of pyrimidine nucleoside transport were made possible by the preliminary establishment of two facts. First, there is no indication of degradation to other metabolites. Intracellular and extracellular fractions of pyrimidine nu-

cleosides incubated with red cells are homogeneous on thin-layer chromatography and identical with untreated nucleoside. In a separate experiment the pyrimidine nucleoside, uridine, was incubated with erythrocytes for several hours. The kinetics of uptake of uridine from the supernatant from this incubation into fresh red blood cells is identical to the uptake of uridine from a freshly prepared solution of the nucleoside. This result is consistent with the observation of LOWY *et al.*[6] that uridine, on incubation with red cells, is not converted to lactate. These data taken together constitute evidence of metabolic stability of pyrimidine nucleosides in the presence of human erythocytes and establish the permeability of their membranes to these compounds. This behavior is in contrast to the marked degradation of purine nucleosides to transportable[5,6] and nontransportable[9] metabolites. Second, the transport of pyrimidine nucleosides is totally inhibited by cold saline with added Hg^{2+}. Time course studies indicate complete retention of the nucleoside within erythrocytes for up to 6 times the time required for analysis of a single sample point.

Preliminary division of red cells preequilibrated with nucleoside into a number of equal portions allows the measurement of the individual time points necessary to define an exit kinetic plot. Direct addition of the cold Hg^{2+} solution to the exit medium allows accurate determination of stop time and consequently v_0. In many cases a decrease of up to 50 % of the original concentration is observed in the first 30 sec. Therefore high accuracy for the time as well as S_i measurements is required. In some competition runs and for the efflux rates determined for the K_m and v_{max} measurements there is less cause for concern for the time axis. Still the individual time point determinations were employed for all exit kinetic runs. The accuracy of v_0, even with these precautions, we estimate to be $\pm$10 %.

The combined inaccuracy in the measurement of v_0's necessary for the K_m and v_{max} determinations give acceptable error limits for these parameters, $\pm$ 0.5 mM for K_m and $\pm$ 1.5 mM/min for v_{max}. The effects of osmolar volume changes have been considered in establishing these limits[10]. Given these data, calculation of v_0 as well as the entire time course of nucleoside efflux when inhibited by a second nucleoside is possible. For the cases reported here the experimental time course for competitively inhibited exits falls within the range calculated from the above parameters.

Several lines of evidence suggest that the transport of pyrimidine nucleosides is by facilitated diffusion. Competition between structurally similar molecules for transport is manifest. The agreement between the calculated and experimental kinetic plots is consistent with a facilitated diffusion as compared to a simple diffusion mechanism. There is no evidence for an active transport system for these nucleosides. There is neither excess accumulation on uptake, nor retention on exit of pyrimidine nucleosides in erythrocytes. Furthermore, the consistency between the initial velocities and time courses for the competition experiments with those calculated from the K_m's and v_{max}'s determined by the method of SEN AND WIDDAS[7] suggests the existence of a single carrier for these nucleosides. These observations also suggest, in a preliminary way, that differences in the mobility of the free carrier and the carrier complexed with nucleoside are small.

The inhibition of pyrimidine nucleoside transport thus far examined permits some preliminary observations on the structural complexity of the carrier. From Fig. 7 it is clear that thymidine uptake is inhibited by sucrose and by inosine, a representative purine nucleoside. Sucrose is impermeable to the erythrocyte membrane

and thus inhibits the pyrimidine nucleoside system without being transported by it. The inhibition of glucose transport in erythrocytes by maltose, a nontransportable disaccharide as in the case of sucrose, is another example of such an inhibition in a facilitated transport system[11]. Inosine, in contrast, is transported across the erythrocyte membrane; however, it is uncertain whether inosine is transported by the pyrimidine nucleoside system or merely inhibits it. The competition between uridine, thymidine, cytosine-β-arabinoside and cytidine show that the functionality and stereochemistry at the 2′ carbon in the pentose moiety and the functionality at the 5 and 6 positions of the pyrimidine ring are unimportant as structural determinants at the transport site. Although thorough evaluation of the structural requirements of the carrier is difficult at this point, focus is placed on the sugar portion of the nucleoside, particularly in view of the sucrose inhibition. It should be noted that we have seen no inhibition of nucleoside transport by glucose or other sugars transported by the glucose system. The fact that the furanose ring and the β-linkage are common to the nucleoside and sucrose may be significant to these findings.

The information on inhibition in the pyrimidine nucleoside transport system and the kinetic characterization of it reported herein do not rule out the possibility of a structurally complex carrier mechanism. The "carrier" may be a composite of two carrier systems functioning independently for furanoside pentoses on the one hand and N^3-substituted pyrimidines on the other. There have been no reports regarding the kinetics of transport for such compounds across erythrocyte membranes nor has any case of a transport system similar in complexity to the one postulated here been documented. Consequently, these preliminary thoughts await extension of our kinetic results to simple furanoside pentoses and to pyrimidine bases similar to those present in the nucleosides examined here. Such information would be fundamental to resolving the issue of the complexity of the pyrimidine nucleoside transport mechanism in human erythrocytes. We are presently in the initial phase of such a study.

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NOTE ADDED IN PROOF: After submission of this paper an important additional reference appeared[12].