Mediated Transport of Nucleosides in Human Erythrocytes

ACCELERATIVE EXCHANGE DIFFUSION OF URIDINE AND THYMIDINE AND SPECIFICITY TOWARD PYRIMIDINE NUCLEOSIDES AS PERMEANTS*

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SUMMARY

Transport of nucleosides in human erythrocytes, previously shown to occur by facilitated diffusion, was further characterized using procedures that measured efflux from cells containing radioactive uridine or thymidine. Efflux of either uridine or thymidine was accelerated several fold in the presence of extracellular uridine or thymidine. Similar apparent kinetic constants for accelerative exchange diffusion of uridine and thymidine and mutual acceleration of efflux indicated that both nucleosides are transported with equal facility by the same mechanism. Specificity of nucleoside transport was investigated by assessing the ability of structurally related compounds to accelerate uridine efflux when added at different concentrations to the extracellular medium. Various pyrimidine ribo- and 2'-deoxyribonucleosides were accepted as permeants by the transport mechanism, which appeared to be less tolerant of modifications in the sugar than in the base portion of the permeant molecule.

Transfer of nucleosides across the plasma membrane in several mammalian cell types is mediated and exhibits the characteristics of "facilitated diffusion" (1-7). Facilitated diffusion, a nonconcentrative form of transport, controls the rate at which particular permeants are transferred across the cell membrane down a concentration gradient (8). Criteria for recognition of transport by facilitated diffusion include saturability of rate, inhibition by compounds structurally analogous to the permeant, and demonstration of "trans" effects. In the latter, movement of permeant from the cis membrane face is influenced by the flow of the same or related permeant from the opposite or trans membrane face.

Accelerative exchange diffusion and counter transport are trans effects that have been observed in studies of facilitated diffusion of glucose (8) and nucleosides (1) in human erythrocytes. Accelerative exchange diffusion, as defined by Stein (8), occurs when the rate of outward transfer of permeant is accelerated by inward transfer of the same or a related permeant present at the opposite membrane face. In counter transport, a permeant, initially equally distributed across the membrane, is driven outward against its own concentration gradient by the inward flow of a related permeant down its concentration gradient. In general, when flow of permeant in one direction is influenced by flow of a related permeant in the opposite direction, both permeants are transported by the same mechanism (8-13).

Previous studies of nucleoside transport in human erythrocytes indicated that rates of uptake of uridine and thymidine were saturable and uptake of both nucleosides was inhibited by several other nucleosides (1, 2). Accelerative exchange diffusion was demonstrated when efflux of labeled uridine or thymidine occurred more rapidly into nucleoside-containing than into nucleoside-free medium; free bases or free sugars did not accelerate efflux. Inosine-driven counter transport of uridine was also demonstrated.

In the present work, accelerative exchange diffusion of nucleosides in human erythrocytes was examined using procedures based on measurements of outward transport of uridine or thymidine, neither of which is phosphorylated or cleaved by erythrocytes (1). Concentration-dependent acceleration of efflux of radioactive uridine by thymidine and of radioactive thymidine by uridine was demonstrated and these experiments formed the basis for use of the accelerative exchange procedure to investigate permeant specificity of nucleoside transport in human erythrocytes.

MATERIALS AND METHODS

Erythrocytes were obtained by centrifuging (1700 × g, 15 min) human blood received from the Red Cross Society Blood Transfusion Service, Edmonton, Alberta, after 21 to 28 days of storage at 4° in acid citrate-dextrose solution A (U.S.P.). After removal of supernatant and white cells, erythrocytes were washed and sedimented (1700 × g, 15 min) three times in TES1-buffered saline (which consisted of 140 mM NaCl, 1.4 mM MgSO4·H2O, and 18 mM TES, at pH 7.4), discarding the upper layer of cell sediment after each wash. Erythrocyte sediments prepared in this way have an extracellular inulin space of 7 to 11% and a total water content of 70 to 75% (1).

Washed erythrocytes were "loaded" by incubating 40 to 50% * This work was supported by the Medical Research Council of Canada and the National Cancer Institute of Canada.

1 The abbreviations used are: TES, A-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid; HTG, 6-[(2-hydroxy-5-nitro-benzyl)thio]guanosine.
cell suspensions in TES-buffered saline containing labeled nucleoside for 40 min at 37°, conditions sufficient to achieve an equilibrium distribution of labeled nucleoside. Loading was terminated by centrifugation at 1700 × g for 15 min. Since nucleosides are not concentrated in erythrocytes (1), internal and external nucleoside concentrations were considered equal at the end of the incubation and the intracellular nucleoside concentration was obtained by measuring extracellular radioactivity.

Efflux of radioactive nucleosides from loaded cells was determined by following the time course of appearance of radioactive nucleoside in the medium using at least four reaction mixtures, one for each point of a given time course. Each reaction mixture was processed to the stage where a sample of cell-free medium was obtained before preparing the next mixture. Efflux was measured at 25° using solutions and reaction vessels that were kept at 25° prior to use. Packed cell sediments were dispersed in 0.25- or 0.50-ml portions (Glaspak disposable glass syringes, Becton, Dickinson and Co.) into 10- or 25-ml flasks and the assay was initiated by rapid addition of 2.5 or 5.0 ml of test medium. Such reaction mixtures were stirred magnetically, and after a timed interval, nucleoside transport in each mixture was terminated by rapid addition of 2.5 or 5.0 ml of TES-buffered saline containing 50 μM HTG. Zero time points were obtained by first adding TES-buffered saline containing HTG, followed 10 s later by addition of test medium.

Immediately after HTG addition, 1- to 2-ml portions of the assay mixture were centrifuged with 5 ml of dibutylphthalate for 11 min at 1500 × g to separate medium from cells (1). Because of differences in specific gravity, centrifugation at room temperature with dibutylphthalate (14) results in a three-layered system with dibutylphthalate separating erythrocytes (lower layer) from the aqueous phase (upper layer). Radioactivity in the aqueous phase of each sample was assayed in triplicate in 5 ml of Bray’s counting solution (15) using liquid scintillation counting. Radioactivity did not appear in the dibutylphthalate phase. The hematocrit of each assay mixture was determined using a capillary tube method.

Efflux, expressed as micromoles per min per ml of packed cells, was calculated after determination of hematocrit, specific activity, and initial rate of appearance of 14C-nucleoside in the incubation medium. Hematocrits from the incubation mixture were used for each rate determination. Specific activities of labeled uridine or thymidine were calculated from measurements of radioactivity and optical density of the medium for each cell sample. Initial rates were obtained from the time course of appearance of radioactivity in the incubation medium; points were taken at 5- to 10-s intervals and straight lines fitted to the data by the method of least squares.

[methyl-14C]Thymidine was purchased from New England Nuclear Corporation and [2-14C]uridine from Schwarz BioResearch. 3-Deazalectidine, 3-deazaauridine, 2′-O-methylcytidine, 3′-O-methylcytidine, 2′,3′-di-O-methylcytidine, 3′-O-methyluridine, 2′,3′-di-O-methyluridine, 4-thiouridine, and 1-methyl-β-D-ribose were gifts from Dr. M. J. Robins, Department of Chemistry, University of Alberta. 3′,5′-Anhydrotimidine, arabinoslyostosine, 6-(methylmercapto)purine ribonucleoside, and 6-methyluridine were generously provided by the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Maryland. HTG was synthesized (16, 17).

Efflux (micromoles/min/ml of packed cells) = initial rate (cpm/min)/specific activity (cpm/μmole) × (1/ml of packed cells).

RESULTS

Inhibition of Nucleoside Transport by 6-[(2-Hydroxy-5-nitrobenzyl)thio]guanosine—A procedure for measurement of initial efflux of uridine or thymidine was developed using the thio-guanosine derivative, ITG, to rapidly terminate nucleoside transport. 4 In Fig. 1 cells loaded with [2-14C]uridine or [methyl-14C]thymidine were loaded with 9.4 mM nonradioactive uridine. HTG was added at 0, 10, or 20 s (see arrows).

by Dr. Brajeshwar Paul1 and obtained from Raylo Chemicals Ltd., Edmonton, Alberta. 4-[2-Hydroxy-5-nitrobenzyl]thio] uridine was synthesized according to the method of Sato and Kanaoka (18). Other compounds were obtained from commercial sources.

Fig. 1. Inhibition of efflux of uridine and thymidine by HTG. Data (expressed as radioactivity per ml of cell-free supernatant) were obtained from duplicate sets of flasks for each concentration of HTG tested. A, efflux from cells loaded with 9.4 mM [2-14C]uridine was measured into medium containing 9.4 mM nonradioactive uridine. HTG was added at 0, 10, or 20 s (see arrows). B, efflux from cells loaded with 9.4 mM [2-14C]thymidine was measured into medium containing 10.0 mM nonradioactive thymidine. HTG was added at 0, 5, 10, or 15 s (see arrows).

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2 S-substituted 6-thiopurine ribonucleosides (2) that inhibit transport of nucleosides are effective stopping agents and are useful in measuring transport at closely spaced intervals (M. A. Pickard and A. R. P. Paterson, unpublished results).
Fig. 2. Accelerative exchange diffusion of uridine and thymidine. Reciprocity of efflux and the concentration of added extracellular nucleoside are plotted in each panel for data obtained during 3 to 4 consecutive days using a single 500-ml unit of blood. Apparent kinetic constants were obtained from lines fitted by the method of least squares; $sV_{\text{max}}$ is expressed as micromoles per min per ml of packed cells. $A$, cells loaded with 6.0 mM [2-14C]uridine were assayed in nonradioactive uridine; $sV_{\text{max}} = 4.3$. $B$, cells loaded with 5.6 mM [methyl-14C]thymidine were assayed in nonradioactive uridine; $sV_{\text{max}} = 5.6$. $C$, cells loaded with 5.5 mM [methyl-14C]thymidine were assayed in nonradioactive thymidine; $sV_{\text{max}} = 7.1$. $D$, cells loaded with 9.9 mM [2-14C]uridine were assayed in nonradioactive thymidine; $sV_{\text{max}} = 4.8$.

14C]thymidine were rapidly suspended in medium containing nonradioactive uridine or thymidine and HTC was added (final concentration of 10 μM) at the times indicated by arrows. For analysis, 0.5 ml samples were removed from the incubation mixtures at timed intervals and immediately centrifuged with dibutylphthalate to obtain cell-free medium. Results in Fig. 1 indicate that 10 μM HTG inhibited efflux of uridine and thymidine almost completely during the 60-s incubation period. Inhibition of efflux was also observed at 6 and 0.6 μM HTG; partial inhibition was observed at 0.06 μM.

The use of HTG in combination with the dibutylphthalate centrifugation method, which separates cells from medium within 30 s (1), permitted sampling at 5- to 10-s intervals. Samples taken after 10 to 15 min of incubation in medium containing HTG indicated that a slow loss of radioactive nucleoside occurred, but at a rate less than 2% of that observed in medium without inhibitor.

To study kinetics of efflux of uridine or thymidine during accelerative exchange diffusion, a unidirectional flux of radioactive permeant must be measured. To determine whether the extracellular volume of medium was sufficient to prevent significant reentry of radioactivity during the incubation period, efflux of uridine was measured in cell suspensions of 5, 10, and 20% (by volume). Data (not shown) indicated that initial rates of outward flow of radioactivity were independent of extracellular volume when cell suspensions of 10% or less were used.

Throughout this work the sampling procedure described in Fig. 1 was used; the time courses of appearance of radioactivity in the medium were consistently linear for 10 to 30 s, indicating unidirectional movement of radioactivity during the period of measurement. Apparent initial rates were calculated from time courses as described in “Materials and Methods.” In Fig. 1 efflux of uridine occurred at an apparent initial rate of 5.5 μmoles per min per ml of packed cells and efflux of thymidine at 6.2 μmoles per min per ml of packed cells.

**Accelerative Exchange Diffusion of Uridine and Thymidine—**In several transport systems with a facilitated diffusion mechanism, the kinetics of unidirectional flux of permeant are described by the Michaelis-Menten equation (8, 19, 20). $V_{\text{max}}$ represents the maximum flux and $K_m$ represents the permeant concentration at which flux is half-maximal; the meaning of these “constants” in descriptions of transport kinetics is unknown. In this description of kinetics of accelerative exchange diffusion, $sV_{\text{max}}$ represents maximum efflux when the concentration of intracellular permeant is above saturation for transport from the internal membrane face and efflux is a function of the concentration of extracellular nucleoside. $sK_m$ is defined as that extracellular concentration of added nucleoside at which efflux is one-half $sV_{\text{max}}$.

To study the dependence of efflux on the presence of nucleoside at the trans membrane face, it was necessary to measure fluxes from cells containing radioactive permeant at concentrations sufficient to saturate the transport mechanism at the cis membrane face. Studies of efflux of radioactive uridine or thymidine at different intracellular concentrations were conducted with extracellular concentration held constant (10 mM) or with extracellular and intracellular concentrations equal. In these experiments efflux was maximal at intracellular concentrations above 4 mM, indicating saturation of transport at the cis membrane face.

In the experiments of Fig. 2 erythrocytes were loaded with excess radioactive uridine or thymidine (5 to 10 mM) and the ability of either nucleoside to accelerate ouflow of the other was tested at several extracellular concentrations. In preparing incubation mixtures, 10 volumes of medium were added to each volume of packed, loaded cells, thereby diluting 125-fold the radioactive permeant present in the extracellular volume of packed cell sediments. The initial extracellular concentrations of nucleoside after addition of nucleoside-free medium to cells loaded with 5 to 10 mM uridine or thymidine were about 0.01 to 0.08 mM, and uridine efflux occurred at a rate of 0.7 to 1.2 μmoles per min per ml of packed cells. Efflux of either nucleoside increased as extracellular concentrations of uridine or thymidine were increased and reached limiting values at extracellular concentrations of about 4 mM.

The acceleration of uridine efflux by thymidine and of thymidine efflux by uridine indicates that transport of both nucleosides is mediated by a single system. Plots of the reciprocals of efflux and of the concentration of added extracellular nucleoside for each of the four possible combinations of uridine and thymidine (Fig. 2) indicate that efflux was a function of the concentration of extracellular nucleoside and was saturable. Values derived from data in Fig. 2, A to D, for the apparent half-saturation constants, $sK_m$, were 0.1 mM; values derived for $sV_{\text{max}}$ were 4.3 to 7.1 μmoles per min per ml of packed cells. Because the apparent kinetic constants for accelerative exchange diffusion are of the same magnitude for all combinations, both uridine and thymidine appear to be transported equally well.

$^{4}$ C. E. Cass and A. R. P. Paterson, unpublished results.
Use of Accelerative Exchange Diffusion to Study Transport Specificity—A procedure using uridine as the radioactive permeant was devised to examine the ability of the uridine-thymidine transport mechanism to accept other nucleosides as permeants. Cells were loaded with excess [2-14C]uridine (6 mM) and efflux of radioactivity was measured in medium containing different concentrations of nonradioactive test nucleoside. As controls, efflux was measured into (a) TES-buffered saline and (b) TES-buffered saline containing the equilibrium concentration of nonradioactive uridine. Efflux into medium that contained test nucleoside was related to efflux into nonradioactive uridine by expressing the former as a percentage of uridine-accelerated efflux, which occurs at maximum rates when the equilibrium concentration is 6 mM.

The procedure is illustrated in Fig. 3 where deoxycytidine was tested as a possible permeant. Comparison of efflux into medium containing different concentrations of deoxycytidine with efflux into TES-buffered saline indicates that uridine efflux was accelerated by extracellular deoxycytidine. These data demonstrate that the rate of uridine efflux was a function of the extracellular concentration of deoxycytidine and was saturable. The half-saturation constant, $s_{K_m}$, was derived from reciprocal plots of efflux of radioactive uridine versus deoxycytidine concentration. Comparison of $s_{K_m}$ for efflux into medium containing deoxycytidine with that for efflux into uridine (Table I) indicates that deoxycytidine is almost as effective an accelerator of efflux as uridine. At saturation, deoxycytidine appears to accelerate efflux of radioactive uridine to a greater extent than extracellular uridine—i.e. $s_{V_{max}}$ for efflux in the presence of deoxycytidine is greater than $s_{V_{max}}$ for efflux in the presence of uridine.

Other pyrimidine nucleosides were first tested for ability to accelerate uridine efflux at 0.5 and 0.0 mM. If acceleration of efflux was observed at these concentrations, the compound was then tested at several additional concentrations in order to derive kinetic constants. For each compound listed in Table I, determinations of efflux were performed using erythrocytes obtained from a single individual. Graphic results for some of the nucleosides listed in Table I are presented in Fig. 4. The data in Table I and Fig. 4 have been related to efflux into nonradioactive uridine by expressing efflux in the presence of test nucleoside as the percentage of efflux in the presence of 6 mM nonradioactive uridine.

When values for $s_{K_m}$ are compared (Table I), extracellular uridine and thymidine appear to accelerate efflux of radioactive uridine at the lowest extracellular concentrations. Other nucleosides with low half-saturation constants are arabinosylcytosine, 2'-deoxyuridine, 5-bromouridine, and 5-bromo-2'-deoxyuridine. Similar $s_{K_m}$ values were obtained for both members of several pairs of ribosyl and 2'-deoxyribosyl nucleosides (compare uridine and 2'-deoxyuridine; cytidine and 2'-deoxycytidine). The compounds of Table I least able to accelerate uridine efflux are dihydrouridine, in which the base moiety dihydouracil differs from uracil in having a puckered non-planar conformation (21), and pseudouridine, in which the ribose moiety is linked to C5 of the pyrimidine ring (22). The ability of dihydouridine and pseudouridine to accelerate efflux of uridine indicates that interaction of permeant with the nucleoside transport system does not require an aromatic base moiety or the N-glycosidic linkage.

Although $s_{V_{max}}$ values for most of the nucleosides capable of accelerating uridine efflux were similar to that of uridine, significantly different values were observed for 5-bromo-2'-deoxyuridine, cytidine, 2'-deoxycytidine, 5-methyl-2'-deoxycytidine, and 5-aminouridine. The pattern of these results was confirmed in other experiments in which uridine efflux was measured at extracellular concentrations of test nucleoside well above saturation for accelerative exchange diffusion.

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**Fig. 3.** Acceleration of uridine efflux by 2'-deoxycytidine. Cells loaded with 6.1 mM [2-14C]uridine were incubated in TES-buffered saline or in TES-buffered saline containing nonradioactive nucleoside. The appearance of radioactivity in the medium versus time is plotted for data obtained during 2 days using a single unit of blood. Panel A: ▲, 6.1 mM uridine; ◇, TES-buffered saline; and ■, 0.5 mM deoxycytidine. Panel B: ○, 6.1 mM uridine; ●, 0.25 mM deoxycytidine; Δ, 1.5 mM deoxycytidine; and ♦, 0.5 mM deoxycytidine.

**Table I**

| External nucleoside                  | $s_{K_m}$ | $s_{V_{max}}$ |
|--------------------------------------|-----------|---------------|
| | mm        |            |
| Uridine                              |           | 0.1           | 103 |
| Thymidine                            |           | 0.1           | 92  |
| 2'-Deoxyuridine                      |           | 0.2           | 105 |
| 5-Bromouridine                       |           | 0.2           | 113 |
| 5-Bromo-2'-deoxyuridine              |           | 0.2           | 78  |
| Arabinosylcytosine                   |           | 0.2           | 106 |
| 2'-Deoxycytidine                     |           | 0.3           | 122 |
| 5-Methyl-2'-deoxycytidine            |           | 0.4           | 141 |
| 5-Aminouridine                       |           | 0.4           | 130 |
| Cytidine                             |           | 0.5           | 121 |
| 6-Methyluridine                      |           | 0.7           | 90  |
| Dihydrouridine                       |           | 0.8           | 98  |
| Pseudouridine                        |           | 0.9           | 103 |

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*When cells were loaded by incubation with [2-14C]uridine, internal uridine concentrations at equilibrium were 5.5 to 6.5 mM. Media containing the equilibrium concentration of nonradioactive uridine were prepared to within 5% of the internal concentration.*
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Fig. 4. Acceleration of uridine efflux by arabinosylcytosine and cytidine. Initial rates of appearance of radioactivity in the medium were determined when cells loaded with 5.5 to 6.0 mM [2-14C]uridine were incubated in media containing different concentrations of nonradioactive nucleoside. Insets contain plots of the reciprocals of efflux (expressed as percentage of maximum uridine-accelerated efflux of uridine) and the concentration of added extracellular nucleoside. A, arabinosylcytosine (ara-C) sKm = 0.4 mM; sVmax = 106%. B, cytidine (Cyd) sKm = 0.5 mM; sVmax = 121%.

Table II

Acceleration of uridine efflux

Efflux from cells loaded with 5.5 to 6.5 mM radioactive uridine into medium containing nonradioactive test nucleoside is compared with efflux into medium containing 5.5 to 6.5 mM nonradioactive uridine. For each preparation the concentration of external uridine was within 5% of the internal uridine concentrations; the concentrations given below are those of the external test nucleoside.

| Nucleoside | Test medium | Uridine efflux into | Uridine | Nucleoside |
|------------|-------------|---------------------|---------|------------|
|            | mM          | percent/min/ml packed cell |
| 3-Deazacytidine | 4.7          | 4.2                  | 4.3     |            |
| 3-Deazauridine | 3.8          | 3.2                  | 4.3     |            |
| 2′-O-Methylcytidine | 6.3          | 2.7                  | 5.2     |            |
| 2′-O-Methyluridine | 5.9          | 2.8                  | 5.2     |            |
| 4-Thiouridine | 5.6          | 2.8                  | 4.9     |            |

Other nucleosides also accelerated uridine efflux but were tested only at a single concentration (Table II). When compared at similar concentrations, extracellular 3-deazacytidine and uridine accelerated efflux of radioactive uridine to the same extent, whereas 3-deazauridine was less effective than either 3-deazacytidine or uridine. 2′-O-Methyluridine, 2′-O-methylcytidine, and 4-thiouridine were also less effective accelerators of efflux than uridine when tested at comparable concentrations. Efflux was determined at several concentrations of extracellular 2′-O-methyluridine and results are compared with acceleration of uridine efflux by 2′-deoxyuridine (Fig. 5). Reasonable estimates of kinetic constants for 2′-O-methyluridine cannot be derived from reciprocal plots of data in Fig. 5; however, assuming a maximum rate of 100%, visual inspection suggests a half-saturation value for 2′-O-methyluridine of approximately 6.0 mM.

Failure of a particular compound to accelerate uridine efflux indicates either that it is not a permeant for the uridine transport system or that, if it is a permeant, it is transported without
measurable effect on efflux of radioactive uridine. Nucleosides and related compounds that had no effect on efflux of radioactive uridine are listed in Table III. These data indicate that (a) nucleosides, free uracil, and free sugars do not accelerate uridine efflux; (b) nucleosides that are present in an ionized form do not accelerate uridine efflux; and (c) modification of the ribosyl or purine ribonucleoside.

The outward flux of radioactive uridine was inhibited by four compounds. To account for accelerative exchange diffusion, the transport mechanism, after releasing outgoing permeant molecules at the external membrane face, reorients or returns to the internal membrane face more rapidly than free carrier. The noncarrier model of Lieb and Stein (28) has proposed a noncarrier model in which transport is accomplished by a membrane-bound oligomeric protein that is simultaneously exposed to intra- and extracellular solutions and capable of undergoing substrate-induced conformational changes resulting in movement of permeant within the oligomeric protein and thence across the membrane.

Kinetic models proposed for facilitated diffusion are based primarily on data obtained from studies of monosaccharide transport in human erythrocytes. The classical carrier model assumes mediation of transport by a carrier capable of moving or rotating within the membrane (9-13, 25-27). Lieb and Stein (28) have proposed a noncarrier model in which transport is accomplished by a membrane-bound oligomeric protein that is dependent on the occupancy state of the several permeant sites on the hypothetical transport protein.

Studies of accelerative exchange diffusion of monosaccharides in erythrocytes (11-13) indicate that when structurally related permeants are transported by the same system of facilitated diffusion, accelerative exchange diffusion occurs when the two permeants are present on opposite sides of the membrane. During accelerative exchange diffusion the transport mechanism, after releasing outgoing permeant molecules at the external membrane face, reorients or returns to the internal membrane face more rapidly in the presence than in the absence of incoming permeant molecules. To account for accelerative exchange diffusion, the carrier model assumes that the carrier-permeant complex moves through the membrane at a greater rate than free carrier. The noncarrier model of Lieb and Stein assumes that the rate of transfer of molecules of permeant within a stationary oligomeric protein is dependent on the occupancy state of the several permeant sites on the hypothetical transport protein.

Studies of accelerative exchange diffusion of uridine and thymidine in human erythrocytes indicate that efflux is dependent on the concentration of extracellular nucleoside and is saturable. Acceleration of efflux of either uridine or thymidine by uridine and thymidine indicates that both nucleosides are transported by the same system of facilitated diffusion, accelerative exchange diffusion the transport mechanism, after releasing outgoing permeant molecules at the external membrane face, reorients or returns to the internal membrane face more rapidly in the presence than in the absence of incoming permeant molecules. To account for accelerative exchange diffusion, the carrier model assumes that the carrier-permeant complex moves through the membrane at a greater rate than free carrier. The noncarrier model of Lieb and Stein assumes that the rate of transfer of molecules of permeant within a stationary oligomeric protein is dependent on the occupancy state of the several permeant sites on the hypothetical transport protein.

The rates of transport of uridine and thymidine at saturation appear to be equivalent; results presented here indicate similar

### Table III

| Compound                  | Test medium | Urine efflux into | Inhibitor | Test medium | Urine efflux into |
|---------------------------|-------------|-------------------|----------|-------------|-------------------|
|                           |             | Test compound     | TS-buffered saline | Concentration | Uridine |
|                           |             | Uridine           | Uridine |
| 3',5'-Anhydrothymidine    | 4.4         | 0.7               | 1.3      | 5.7         |
| 6-Azauridine              | 0.7         | 1.1               | 1.1      | 4.5         |
| Cytidine 5'-phosphate     | 5.6         | 1.2               | 0.9      | 4.4         |
| Orotidine                 | 0.5         | 1.5               | 0.9      | 4.9         |
| D-Ribose                  | 5.8         | 1.5               | 1.2      | 3.6         |
| 1-Methyl-β-D-ribose       | 5.3         | 1.1               | 1.1      | 5.5         |
| 3'-O-Methylcytidine       | 5.9         | 1.0               | 1.0      | 5.2         |
| 2',3'-Di-O-Methylcytidine| 5.5         | 1.3               | 1.0      | 5.1         |
| Uridine 5'-phosphate      | 6.0         | 0.9               | 1.2      | 5.2         |
| Uracil                    | 6.1         | 1.0               | 0.9      | 4.4         |

### Table IV

| Inhibitor                  | Test medium | Urine efflux into |
|----------------------------|-------------|-------------------|
|                           |             | Concentration     | Uridine |
| 2',3'-O-Isopropylidene-uridine | 6.1         | 0.1               | 0.8      | 4.5         |
| 2',3'-Di-O-methyluridine   | 5.8         | 0.4               | 0.9      | 5.1         |
| 6-(Methylthio)-purine ribonucleoside | 5.9         | 0.4               | 0.9      | 5.3         |
| 4-(2-Hydroxy-5-nitrobenzyl)thio-uridine | 0.1         | 0.9               | 9.5      | 5.4         |

For each preparation the concentration of external uridine was within 5% of the internal uridine concentration; inhibitor concentrations are given below.

DISCUSSION

Kinetic models proposed for facilitated diffusion are based primarily on data obtained from studies of monosaccharide transport in human erythrocytes. The classical carrier model assumes mediation of transport by a carrier capable of moving or rotating within the membrane (9-13, 25-27). Lieb and Stein (28) have proposed a noncarrier model in which transport is accomplished by a membrane-bound oligomeric protein that is simultaneously exposed to intra- and extracellular solutions and capable of undergoing substrate-induced conformational changes resulting in movement of permeant within the oligomeric protein and thence across the membrane.

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Studies of accelerative exchange diffusion of uridine and thymidine in human erythrocytes indicate that efflux is dependent on the concentration of extracellular nucleoside and is saturable. Acceleration of efflux of either uridine or thymidine by uridine and thymidine indicates that both nucleosides are transported by the same mechanism and kinetic studies suggest little difference in affinity of the transport mechanism for either nucleoside. The rates of transport of uridine and thymidine at saturation appear to be equivalent; results presented here indicate similar
values for $\dot{V}_{\text{max}}$ for efflux and results from studies of uptake of uridine and thymidine (1) and from the kinetics of equilibrium exchange diffusion of uridine and thymidine suggest similar maximal rates of influx for both nucleosides.

A procedure based on the dependence of uridine efflux on the concentration of permeant present at the external membrane face was used to examine the chemical specificity of the nucleoside transport system in erythrocytes. Among pyrimidine nucleosides that act as permeants in the uridine-thymidine transport system are such diverse structural analogs of uridine as 2'-deoxyuridine, 5-bromouridine, 6-aminouridine, 6-methyluridine, 3-deazauridine, dihydrouridine, pseudouridine, and 4-thiouridine. The C-glycosides, pseudouridine and formycin B (1), are permeants indicating that the N-glycosidic linkage is not crucial for interaction of permeant with the transport mechanism. Earlier studies (1) demonstrated increased efflux of radioactivity when cells loaded with uridine were incubated in media containing 1.0 to 2.0 mm adenosine, inosine, or guanosine, indicating that purine nucleosides are also permeants. Failure of 6-aza-uridine and orotidine to accelerate uridine efflux suggests that the presence of charged groups on the base is not accommodated by the transport mechanism.

The transport mechanism is less tolerant of changes in the sugar portion of the permeant molecule than in the base. There appears to be little tolerance for substitution on the 2'- or 3'-hydroxyl groups since methylation at either position greatly reduces the ability of uridine or cytidine to accelerate efflux and the 2',3'-substituted uridine derivatives inhibit efflux of uridine when assayed in the absence of external uridine. However, the 2'-hydroxyl group is not essential for interaction of permeant with the transport mechanism since several pairs of pyrimidine ribo- and deoxyribonucleosides that differ only at the 2' position have comparable $K_{\text{m}}$ values; furthermore, acarbose, 2'-deoxyriboinosine, in which the 2'-hydroxyl group is cis to the glycosidic linkage, promotes efflux as effectively as 2'-deoxyuridine. These results and structure-activity studies with purine nucleoside permeants support the conclusion that the sugar moiety is more important than the base in recognition of permeant by the transport mechanism.

HTG and 6-(methylthio)purine ribonucleoside inhibit uridine efflux in the presence and absence of extracellular uridine, and HTG is inhibitory at concentrations as low as 0.06 µM. That HTG and other related S-substituted 6-thiopurine ribonucleosides (2) inhibit nucleoside transport suggests that addition of bulky hydrophobic groups to the sulfur atom of 6-thiopurine ribonucleosides imparts a high affinity for the nucleoside transport mechanism. Inhibition of uridine efflux by the uridine derivative, hydroxyaminobenzylthiouridine, was much less effective than inhibition by HTG, suggesting greater affinity of the inhibitory site or sites on the transport mechanism for purine ribonucleosides than for pyrimidine ribonucleosides.

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