Transport of Uridine in Human Red Blood Cells

Demonstration of a Simple Carrier-Mediated Process

Z. IOAV CABANTCHIK and HAGAI GINSBURG

From the Biophysics Group, Institute of Life Sciences, The Hebrew University, Jerusalem, Israel. Dr. Cabantchik's present address is the Laboratory of Kidney and Electrolyte Metabolism, National Institutes of Health, Bethesda, Maryland 20014.

ABSTRACT The kinetic properties of the mediated transport of uridine in human erythrocytes are investigated. Different methodological procedures are used to acquire a complete kinetic description of the system. Under equilibrium-exchange (ee) conditions the respective values of maximum velocity and Michaelis constant at 25°C are 7.54 ± 0.45 mM/min and 1.29 ± 0.11 mM. Under zero-trans (zt) conditions the kinetic properties of efflux (to) differ significantly from those of influx (oi), revealing a 4:1 asymmetry in the system: V_t0 = 1.98 ± 0.31 mM/min and K_{t0} = 0.40 ± 0.12 mM; V_{o1} = 0.53 ± 0.038 mM/min and K_{o1} = 0.073 ± 0.069 mM. These data are analyzed in terms of the simple carrier model as formulated by Lieb and Stein (1974, Biochim. Biophys. Acta. 373:178). Using this model and the data of equilibrium-exchange and zero-trans we have predicted the half-saturation constants in infinite-cs conditions (ic) and compared them with the experimental values (given in parentheses): K_{i0} = 0.291 (0.252) mM and K_{i1} = 1.08 (0.937) mM. This indicates the internal consistency of the simple carrier model for uridine transport. Furthermore, application of several rejection criteria developed for the simple carrier failed to indicate lack of fitness of the model in the present case. From the analysis of kinetic data we infer that the movement of the unloaded carrier is the rate-limiting step in transport of uridine. From the value of 10^6 uridine carrier molecules per cell we calculate the turnover rate for uridine transport to be 7,600 molecules/min for influx and 35,000 molecules/min for efflux (both at 25°C). The present work provides a unique example of an asymmetric transport mechanism which is fully consistent with the predictions of a simple carrier model. The mechanism is discussed in terms of current concepts of membrane structure.

INTRODUCTION

The human red blood cell has for many years provided a classical experimental object for the investigation of transport phenomena in biological membranes. Among the various transport systems present in this cell membrane, the system responsible for glucose movement has been one of the most thoroughly investigated, particularly in regard to kinetic description of molecular events underlying...
ing the transport process (1, 2, 3). This system has also provided the favorite object for the application of new methodological approaches to transport and for the testing of theories of membrane transport. However the system for which most carrier theories were formulated has recently been subjected to new methodologies which provide a body of kinetic evidence that seems incompatible with a simple carrier mechanism (1, 3). Thus, in order to characterize a transport system in terms of a simple carrier mechanism, it is necessary to subject it to a complete analysis of kinetic data obtained by all required methodologies.

We have undertaken the present study with the aim of describing the kinetic properties of the uridine transport system in human red blood cells using several experimental approaches to test whether the conventional carrier model can be applied to the transport of nucleosides. The experimental procedures performed in both directions included: (a) equilibrium-exchange (ee) for the measurements of unidirectional fluxes when the substrate concentration is identical on both sides of the membrane; (b) zero-trans (zt) for the measurements of net fluxes when the concentration of substrate at the trans side of the membrane is maintained at zero while that at the other is varied; and (c) infinite-cis (ic) for the measurements of net fluxes when the substrate concentration at the cis side of the membrane islimitingly high while that at the trans side is varied.

It has been known for some years that a single transport system which facilitates the diffusion of uridine and other nucleosides is present in human red blood cell membranes (4, 5, 6). It has also been shown that pyrimidine nucleosides do not undergo chemical changes within human red blood cells. Thus, the study of uridine transport can be carried out independently of subsequent metabolic conversions (5). In a preliminary work, Pickard and Paterson (7) presented some evidence which could have pointed towards the fact that the kinetic parameters of uridine exchange efflux differ substantially from those of zero-trans influx. These parameters apparently also differ from those obtained by the infinite-cis procedure (4). On the basis of this observation it was proposed that uridine and glucose might be transported by mechanisms which display similar properties (5). However, the aforementioned data could not be directly compared, first, since they were obtained at significantly different temperatures, and second, since the experimental methods did not allow derivation of kinetic data with sufficiently high precision.

In the present study we have conducted a careful examination of uridine fluxes in human red blood cells. The experimental procedures which were tested on both influx and efflux of uridine included exchange, zero-trans, and infinite-cis (8). The kinetic parameters derived from the latter were checked to see whether they are in line with the prediction of a simple carrier mechanism. We have conducted tests which rule out any contribution of unstirred layers at the inner or outer surface of the red blood cell membrane. Finally, we have applied several rejection criteria which enable us to test the simple carrier model. The analytical part of this work has been based primarily on the steady state analysis of a simple carrier as recently formulated by Stein and Lieb (8) and Lieb and Stein (9). This treatment allows not only the characterization of the transport system in terms of four independent parameters, but also the interpretation
of these parameters in terms of molecular rate constants. It has also the advantage of making no assumptions as to equilibrium or symmetry. In this study we demonstrate that uridine transport can be fully described by a simple carrier mechanism. This mechanism displays asymmetric properties inasmuch as the maximal velocity of influx $V_m$ (zero-trans in) is about 4.3 times faster than the maximal velocity of efflux $V_m$ (zero-trans out) at 25°C. (The corresponding $V_m/K_m$ values are the same in both directions.) This model is also discussed in terms of current concepts of structure of red blood cell membrane components.

**EXPERIMENTAL METHODS**

**Chemicals**

Uridine, 6-mercaptopurineriboside (mercaptoinosine = MI), and 6-mercaptopguanosine (MG) were purchased from Sigma Chemical Co., St. Louis, Mo., hydroxynitrobenzylbromide and $p$-nitrobenzylbromide from Aldrich Chemical Co., Inc., Milwaukee, Wis. [3H]Uridine (10 Ci/mmol) was procured from the Israel Atomic Energy Agency, Isotope Laboratory, Nuclear Research Center, Negev. The nitrobenzyl derivatives of MI (i.e. $p$-nitrobenzyl-S-mercaptoinosine = NBMI and hydroxynitrobenzyl-S-mercaptoinosine = HNBMG) were prepared by a modification of a previously published method (10). Briefly, equimolar amounts of the respective benzyl bromides and mercaptonucleosides were dissolved in redistilled $N,N$-dimethylformamide (Merck-Darmstadt, West Germany) containing equimolar amounts of anhydrous $K_2CO_3$. The reaction was carried out under anhydrous conditions for 2 h at 60°C and was followed immediately by filtration of insoluble salts, removal of solvent by flash evaporation, and trituration. Traces of dimethylformamide were removed by repeated coevaporations in vacuo with ethanol at 40°C. The powder was washed several times with diethylether and finally with petroleum ether and kept desiccated in vacuo at 4°C. The properties of the final products (IR, UV and visible spectra, melting points, and chromatography properties) are essentially similar to those published in a recent work (11).

**Preparation of Cells**

Recently outdated human blood was obtained from Hadassah Medical School Blood Bank, Jerusalem. The cells were obtained free of buffy coat and washed four times with isotonic phosphate-buffered saline, pH 7.4, at room temperature. For studies of uridine efflux, cells at a 5% hematocrit suspension were loaded with uridine to various predetermined concentrations by incubation at 37°C, generally for 1 h. The loaded cells were subsequently packed and a small aliquot of $[^3H]$uridine (1 mCi/ml buffer) was added and the cells were incubated for another hour at 37°C and finally packed.

**Efflux Measurements**

As a general practice, efflux of uridine was assayed by measuring the appearance of label in the washout medium by the filter technique originally devised by Mawe and Hempling (12). Filters (0.45 μm) and prefilters were mounted in 25-mm diam holders (Sartorius GmbH). Holders were connected to 5-ml Luer-lock syringes and tested for air tightness. Preloaded erythrocytes were packed by spinning in a Beckman Microfuge (Beckman Instruments, Spinco Div., Palo Alto, Calif.) for 5 min and aspirated into a length of Teflon tubing connected to a syringe prefilled with 1-2 ml of the washout medium. At
time zero the erythrocytes were flushed instantaneously into a thermostated (25°C) beaker containing vigorously stirred washout medium (magnetic spinbar, 300 rpm). Homogeneous mixing is thus achieved within 1–2 s. The final hematocrit was 0.01%. At the desired time intervals, samples of 0.5–1.0 ml were aspirated through the filters and the filter holders were immediately disconnected from the syringe to avoid further leakage. With some practice the time needed for withdrawal of the sample could be reduced to 1 s; this lag time was added to the “elapsed time.” To 250 μl of the sampled medium were added 2.5 ml of scintillation fluid (toluene 67% vol/vol; Triton X-100 33% vol/vol; 2,5-diphenyloxazole, 0.55% wt/vol; 1,4-bis-2-(4 methyl-5-phenyloxazolyl), 0.01% wt/vol) and radioactivity was assayed with a Packard Instrument Scintillation Spectrometer (Model 3380, Packard Instruments, Inc., Downers Grove, Ill.). Counts were usually normalized for quenching.

Influx Measurements

A 0.2-ml vol of solution consisting of isotonic buffered saline and the desired concentration of tritium-labeled uridine were added at time zero to a 20-μl volume of packed washed erythrocytes. The reaction system was under vigorous vortexing. At the desired time interval, 8 ml of stopper solution (isotonic buffered saline plus either 4 μM NBMI or 20 μM HNBMI at room temperature) were added under continued vortexing. The resulting suspension was spun down at 7,000 rpm for 10 s and the supernate was aspirated. The cell pellet was resuspended in 8 ml of stopper solution and spun again, and the supernate was discarded. A dilution of about 10⁴-fold of the extracellular medium was thus obtained. The cell pellet was dissolved in 3 ml of hemolyzing solution (CaCl₂, 4mM; NH₄OH, 0.25%; Triton X-100, 50 ppm). To 1 ml of the hemolyzate was added 0.1 ml of 100% wt/vol trichloroacetic acid and subsequent to centrifugation 250 μl were taken for counting the radioactivity. The remaining hemolyzate was used for measurement of hemoglobin absorbance at 540 nm using a Gilford 2400 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). Nonspecific absorption of uridine to the cells was assayed by exposing cells pretreated with stopper solution to the loading solution for 2 s and then stopping and washing according to the general procedure. Zero time values thus obtained were subtracted from values at different sampling times to take care of contributions from nonspecific binding and/or contamination.

Calculations

Zero-trans Influx (\( \dot{\Delta} \))

The internal concentration (\( C_i \)) of [\(^3\)H]uridine at each sampling time was calculated according to the following equation:

\[
C_i = H \cdot \frac{D - B}{O_1 \cdot S_a} \cdot L
\]

(1)

where

\[
H = \frac{OD_{st} \cdot 25}{HCT_{st} \cdot 50}
\]

(2)

\( OD_{st} \) is the optical density at 540 nm of 50 μl of cell suspension at hematocrit \( HCT_{st} \), which was hemolyzed in 25 ml of hemolyzing solution (see previous section). \( D \) is the disintegrations per minute (dpm) of the sample, \( O_1 \) is the optical density at 540 nm of the same sample, \( B \) is the dpm of background, and \( S_a \) is the specific activity in dpm per microliter of loading medium per μM uridine concentration given in mM. \( C_i \) is given in millimoles per
liter cell water. $L$ is a constant (i.e. correction factor) which takes into account: (a) the dilution due to addition of TCA (10% final); (b) the aliquot of solution taken for counting (0.25 ml); and (c) the cellular water fraction under the experimental conditions (0.7). Thus $L = 4 \cdot 1.11/0.7$. The rate of uptake $v$ is obtained directly from the slope of $C_i$ against time $t$. The intercept of this line coincided precisely with the $t = 0$ values obtained as previously described. The Michaelis constant $K_m$ and the maximum velocity $V_m$ are obtained by linear least squares regression analysis of either $v/S$ against $v$ or $S/v$ against $S$ plots (S being the external uridine concentration).

**Zero-trans Efflux ($\frac{dpm}{dt}$)**

When one uses the filter technique, a linear regression of net $dpm$ against $t$ yields an intercept $I$ at $t = 0$ which represents the initial extracellular radioactivity. A linear regression of $(dpm_t - I)/(dpm_{m} - I)$ against $t$ gives a slope which is equal to $v/S$. The value of $dpm_{m}$ is obtained either by adding TCA to the reaction vessel (final concentration 10%) or by letting the system equilibrate (for at least 30 min at 37°C). The calculation relies on the fact that efflux was linear with time as long as $C_i$ was reduced no more than 20% of the initial value. A correct choice of experimental conditions (time of sampling, specific activity, and concentration of cells) allowed us to obtain reliable and reproducible results. The $K_m$ and $V_m$ values were calculated by linear regression of $S/v$ against $S$ plots. The internal concentration of uridine $C_i$ was assayed at different times by the stopper method described before under Influx Measurements. The $C_i$ values were calculated according to Eq. (1) or by extrapolation assuming that $D/0$ (dpm/optical density) at $t = 0$ is equivalent to the original concentration of uridine to which cells were preloaded. Similar results were obtained by both methods of calculation.

**Equilibrium-Exchange Efflux ($\frac{dpm}{dt}$)**

For equilibrium exchange efflux we know that:

$$\ln \left( \frac{C_m - C_i}{C_m} \right) = -k \cdot t,$$

where $C_i$ and $C_m$ are the respective concentrations or radioactivities of substrate appearing in the external medium at time $t$ and time infinity (i.e. time of full equilibration) while $k$ is the corresponding rate constant.

The slope of the natural logarithm of the fractional radioactivity remaining in the cells $\ln ((C_m - C_i)/C_m)$ plotted against time yields directly the rate constant $k$ for exchange which is equal to $v/S$. Thus it is convenient to use directly the dpm of labeled uridine appearing in the external medium at each sampling time, then to normalize them according to the dpm at $t = \infty$ yielding the fraction exit $F$, and finally to calculate the slope of $\ln (F)$ vs. $t$ by linear regression. The constants $K_m$ and $V_m$ are then computed from conventional plots as detailed for zero-trans influx.

**Equilibrium-Exchange Influx ($\frac{dpm}{dt}$)**

The cellular concentration of labeled uridine was calculated as for the zero-trans influx. Initial rates were taken for the calculation of $V_m$ and $K_m$ as detailed for the zero-trans methods. This method was preferred over that described for exchange efflux for technical convenience, although both methods are equally reliable.

**Infinite-cis Efflux ($\frac{dpm}{dt}$)**

The initial concentration of radioactive substrate to which cells were loaded is equated with the dpm measured at time zero. The $C_i$ values at different times are obtained by
relating the corresponding dpm values with the above values at time zero. The $K_{m}$ value obtained by this procedure is defined as that external uridine concentration which reduces the net maximal efflux by one-half. The rate of net efflux is the difference between the unidirectional efflux at infinite uridine inside (i.e. $V$) and the unidirectional influx as given by

$$v_{net} = V - \frac{V S_o}{K + S_o},$$

where $S_o$ is the external substrate concentration (trans-side). From Eq. (4) we derive

$$\frac{1}{v} = \frac{1}{V} + \frac{S_o}{K \cdot V}. \quad (5)$$

By plotting $1/v$ vs. $S_o$, the intercept on the x axis yields directly $-K$, which we define as $K_{m}$.

**Infinite-cis Influx ($S_o$)**

The intracellular concentrations $C^i$ at different sampling times $t$ were calculated either as detailed for the zero-trans influx procedure or, alternatively, by equating the concentration of uridine in the preloading medium to the dpm found at time zero. This is based on the fact that the preloading and the uptake media have the same specific activity of $[\alpha H]$uridine. The initial rate of uptake was obtained by linear regression of $1/v$ vs. $S_i$ plots ($S_i$ is the cellular concentrations of substrate) and the $K_m$ was derived as for the infinite-cis efflux procedure (see Eq. [5]).

**RESULTS**

**Efficiency of Stopping and Washing Procedure**

The study of cellular transport and its kinetic parameters relies on the precise determination of the instantaneous concentration of substrate within the cells at defined periods of time. In order to accomplish this determination it was essential to apply a stopping technique which led to a fast, effective, and persistent inhibition of uridine fluxes. The stopping technique, which consists of rapidly adding an inhibitor to the cell suspension at room temperature, is evaluated in the following way. The time course of $[\alpha H]$uridine exchange efflux was obtained by measuring the isotope egress from preloaded cells by the filter technique (Fig. 1). After the flux has been followed for an initial 30 s period, either NBMI (1 μM) or HNBMG (10 μM) is added and efflux measured for a total 240 s-period. The extrapolation of the uridine egress back to the time of addition of inhibitor indicates that the stopping is virtually instantaneous (less than 2 s at 25°C in a well stirred suspension). If the egress of $[\alpha H]$uridine from cells is into medium free of uridine, the inhibitor is equally potent as a stopper and its effects last for longer periods of time (10 min).

For measurements of instantaneous concentrations of substrate within the cells it was necessary to stop the fluxes efficiently at defined times and to free the cells from extracellular radioactive contamination. Stopping was accomplished by a 40-fold dilution of the substrate in the outer medium and by a simultaneous effect of the inhibitor (i.e. stopping-dilution medium containing an appropriate concentration of inhibitor). The next step consisted of a washing procedure which allowed the retention of the intracellular $[\alpha H]$uridine during removal of
the extracellular material (Table I). With two such washes the extracellular component can be completely eliminated. However, if the washing is omitted altogether and the "stopped" cells are obtained free of supernate by centrifugation over dibutyl phthalate (6), the contamination by extracellular material can

![Graph](image)

**Figure 1.** Efflux of [³H]uridine from preloaded cells assayed by the filter technique. The radioactivity in the external medium corrected for extracellular radioactivity of the cell pellet is plotted against time. The symbols ○ and ● refer to two different control experiments. At the time indicated by the arrow (30 s), stopper is added to the system and the extracellular radioactivity is sampled after an indicated interval. △, HNBMG, final concentration 10 μM. ◇, NBMI, final concentration 1 μM.

**TABLE I**

| No. of washing | 1   | 2   | 3   | 4   | 5   |
|---------------|-----|-----|-----|-----|-----|
| Sample 1      | 42,029 | 30,566 | 29,579 | 28,950 | 28,412 |
| Sample 2      | 41,122 | 31,699 | 30,003 | 30,396 | 29,821 |

Packed cells (20 μl) were mixed with 200 μl of 0.1 mM labeled uridine in PBS at 25°C. After 30 min, when equilibrium was achieved, 8 ml of PBS containing 1 μM NBMI were added with vigorous mixing. The suspension was centrifuged at 5,000 rpm for 5 s, the supernate was discarded, and dpm remaining in the cell pellet were determined as detailed in Experimental Methods. This constitutes the no. 1 washing treatment. Other samples were resuspended in 8 ml of stopping solution, recentrifuged, etc., up to five washings. dpm values corrected for the optical density of the sample are given as a function of the number of washings.

amount to more than 80% of the intracellular material (unpublished observations).

**Equilibrium-Exchange Influx and Efflux**

In equilibrium-exchange experiments, the substrate concentration is the same at the two faces of the membrane and the unidirectional flux of isotopically labeled substrate is
measured at different substrate concentrations. This is the only experimental procedure
that can be described by a simple Michaelis-Menten formalism of the simple carrier,
regardless of possible effects of unstirred layers (9). This procedure has the additional
advantage of yielding accurate values of kinetic parameters of transport.

By using the Michaelis-Menten formalism and a conventional transformation of the
first-order reaction \( v = V_s S / (K + S) \) into any reciprocal form which gives a straight line, it
is possible to obtain the kinetic parameters \( V \) and \( K \) by linear least squares analysis.
Cleland (13) has shown that in order to obtain accurate estimates of kinetic parameters the
experimental data must be properly weighed. This is most important if the variances of
the measured velocities are not constant and inasmuch as each linearization method
emphasizes results related to different ranges of substrate concentrations. However, if
two such linearization methods yield similar kinetic parameters, it can be safely concluded
that the variances are essentially constant throughout the experimental concentration
range. The results shown in Fig. 2 A, B for uridine exchange efflux comply with this
condition.

At least eight different substrate concentrations were used in each experiment and the
velocities were obtained as indicated in Experimental Methods. Linear regression analysis
of \( \ln \left( \frac{C_r - C_t}{C_r} \right) \) against \( t \) invariably showed very high correlation coefficients.
Although the extracellular label at \( t = 0 \) could be obtained by linear extrapolation to \( t = 0 \)
and subtracted accordingly, this calculation was found unnecessary for the computation
of the rate constants under the prevailing experimental conditions.

The results of all equilibrium exchange flux experiments were subjected to the
following analyses: linear least squares fit of \( S/v \) against \( S \) and of \( v/S \) against \( v \) (Fig. 2, B
and A, respectively, for efflux, and Fig. 4 for influx), and nonlinear least squares analysis
based on the Levenberg-Marquand technique (Fig. 3). Although the \( S/v \) against \( S \) plot
emphasizes data obtained at relatively high concentrations while the \( v/S \) against \( v \) plot
emphasizes the opposite, the kinetic parameters derived from them are essentially
identical (Table II), coinciding also with the parameters derived by the nonlinear least
squares procedure.

**Zero-trans Influx and Efflux**

In the zero-trans procedure, the substrate concentration at one face of the membrane is
kept at zero while that at the other face is varied. A comparison of kinetic data of zero-
trans efflux and influx permits one to draw conclusions with regard to the degree of
asymmetry of the transport system.

For the zero-trans influx experiments we used loading solutions containing constant
specific activities of \(^{3}H\)uridine. These solutions were obtained by serial dilutions of a
solution containing the highest uridine concentration tested. Since the rate of mixing cells
with medium was very fast (<1 s) it was also possible to sample at time intervals of 3 s.
These times were sufficiently short to allow the estimation of initial rates even with the
lowest substrate concentration used. Nonspecific adsorption was determined either by
extrapolating cellular dpm (normalized for the optical density of the sample) back to \( t = 0 \)
or by measuring uptake of label by cells which have been pretreated with inhibitor. Both
methods yielded virtually the same values which ranged from 5% at low substrate
concentrations to 40% at the highest concentrations used. The method of calculating
internal concentration of substrate (see Experimental Methods) was also validated by
estimating the intracellular substrate in cells which were allowed to equilibrate with
external medium. The kinetic parameters were estimated by a linearization method (\( S/v \)
against \( S \)) (Fig. 5 and Table III).

The zero-trans efflux was measured by the filter technique on cells preloaded to the
desired substrate concentration. Equilibrium values were obtained either by sampling the cell suspension after very long periods of incubation (compared with $t_0$) or by lysing the cells with TCA. Efflux rates were obtained from linear regression analysis.

Extrapolation of the external dpm back to $t = 0$ allowed the estimation of extracellular material. Since the latter could be very significant at short sampling intervals, it was found necessary to determine them with high precision. Simultaneous measurement of zero-

\[ \begin{align*}
A \quad & \text{EE-OUT} \\
\begin{array}{c}
V/S (\text{min}^{-1}) \\
0 & 3 & 6 & 9
\end{array} \\
\begin{array}{c}
\text{v} \text{mM} \\
0 & 3 & 6 & 9
\end{array}
\end{align*}

\[ B \quad \text{EE-OUT} \]

\[ \begin{align*}
S/V (\text{min}) \\
-5 & 0 & 5 & 10 & 15 & 20
\end{align*} \]

\[ [u.] \text{mM} \]

**Figure 2.** A representative experiment of equilibrium-exchange efflux of $[^3\text{H}]$-uridine. Efflux at different uridine concentrations was obtained by the filter technique using cells preloaded with $[^3\text{H}]$uridine to the desired concentration (0.1-20 mM). Rates were calculated as detailed in Experimental Methods. A, $v/S$ against $v$ plot yielding $V_{ee} = 8.24 \pm 0.83 \text{ mM/min}, K_{ee} = 1.27 \pm 0.11 \text{ mM},$ and correlation coefficient $r = 0.979$. B, $S/v$ against $S$ plot of the same data yielding $V_{ee} = 8.23 \pm 0.19 \text{ mM/min}, K_{ee} = 1.31 \pm 0.09 \text{ mM},$ and $r = 0.999$.

\textit{trans} efflux by filter and stopping techniques yielded essentially the same results. The filter technique is nevertheless advantageous and less susceptible to experimental errors inasmuch as it involves less dilution and sampling. The kinetic parameters derived for the efflux procedure are given in Table III. The values obtained show a significant difference from those of influx values, indicating an asymmetry in the transport mechanism.

**Infinite-cis Influx and Efflux**

The kinetic parameters derived from the exchange and zero-\textit{trans} procedures are suffi-
FIGURE 3 (above). Equilibrium-exchange efflux of [3H]uridine. The data used in Fig. 2 were analyzed by the nonlinear least squares method using the Levenberg-Marquardt technique. The line was drawn by the computer using the optimum values given by the analysis: $V_{\text{ee}} = 8.27 \pm 0.24 \text{ mM/min}$ and $K_{\text{ee}} = 1.32 \pm 0.14 \text{ mM}$.

FIGURE 4 (below). A representative experiment of equilibrium-exchange influx of [3H]uridine. Cells were preloaded with uridine to the desired concentration (0.04-10.0 mM) and influx of [3H]uridine was measured by the stopper technique. Data are shown in terms of the linearized representation of $S/V$ against $S$. The linear least squares analysis of this experiment yielded the following values: $V_{\text{in}} = 12.15 \pm 0.42 \text{ mM/min}$; $K_{\text{in}} = 1.28 \pm 0.14 \text{ mM}$. The correlation coefficient was $r = 0.996$.

The data fully to describe a simple carrier mechanism and test it by various rejection criteria (14, 9). However, for the sake of internal consistency of the kinetic analysis, a third experimental method, the infinite-$c/s$ procedure, was also applied. The last, originally introduced by Sen and Widdas (15), consists of measuring net fluxes by setting the $cis$ side at high substrate concentration relative to $K_m$ while at the $trans$ side the substrate concentration is varied. $K_m$ is defined as that $trans$ concentration which reduces the
TABLE I

COMPARISON OF VARIOUS ANALYTICAL TECHNIQUES FOR DERIVATION OF KINETIC PARAMETERS

| Method          | Parameter | \( \frac{v}{S} \text{ against } v \) | \( \frac{S}{v} \text{ against } S \) | LSQ  |
|-----------------|-----------|--------------------------------------|-------------------------------------|------|
|                 | \( V \pm SE \)          | 8.24±0.83                           | 8.23±0.092                          | 8.27±0.24 |
|                 | \( K \pm SE \)          | 1.27±0.11                           | 1.31±0.092                          | 1.32±0.14 |

In this case the results of two equilibrium exchange efflux experiments in the concentration range of 0.025–20.0 mM were lumped together and analyzed by linear least square regression analysis of \( \frac{v}{S} \text{ against } v \) and of \( \frac{S}{v} \text{ against } S \), and by the nonlinear least squares method (LSQ program of the Hebrew University Computation Center). \( V \) is given in mM/min. \( K \) is given in mM.

**FIGURE 5.** A representative experiment of zero-trans influx (A) and efflux (B) of \(^{3}H\)uridine as a function of uridine concentration. Data are shown in terms of the linearized representation of \( \frac{S}{v} \text{ against } S \). The linear least squares analysis of these representative experiments yielded the following values: \( V_{in} = 0.78 \pm 0.03 \) mM/min. \( K_{in} = 0.073 \pm 0.069 \) mM (compilation of two experiments). The correlation coefficient was \( r = 0.988; V_{in} = 1.70 \pm 0.051 \) mM/min; \( V_{in} = 0.488 \pm 0.059 \) mM; \( r = 0.997 \).

maximum net flux by one-half and is obtained by plotting \( \frac{1}{v} \text{ against the trans concentrations} \) (see Experimental Methods). This plot yields a straight line with an intercept on the \( x \)-axis equal to \( -K_m \). This procedure is appropriate for either efflux or influx.

The results of uptake of \(^{3}H\)uridine with time from an external infinite concentration into cells containing varying concentrations of substrate \( S \) (all the uridine solutions have the same specific activity) are depicted in Fig. 6. As the internal concentration of uridine
Si increases, the net influx is slowed down accordingly. The linearized 1/v against Si plot for influx is shown in Fig. 7. The kinetic parameters obtained by linear regression analysis are presented in Table IV, which also includes the theoretical $K_m$ parameters computed from the two previous methods (see Discussion and Appendix). The results of the infinite-cis efflux experiments are shown in Figs. 8 and 9.

### Table III

| Procedure | V | K | $V/K$ |
|-----------|---|---|-------|
| ee-(io)   | 7.85±1.218 | 1.31±0.092 | 6.00±1.02 |
| ee-(oi)   | 7.16±0.364 | 1.28±0.142 | 5.59±0.68 |
| zt-(io)   | 1.98±0.310 | 0.40±0.122 | 4.91±1.67 |
| zt-(oi)   | 0.53±0.038 | 0.073±0.063 | 7.27±6.29 |

Data presented are weighted means and SE of several experiments in each procedure, derived from S/v against S linear least squares analysis.

**Figure 6.** Infinite-cis influx of [H]uridine. Net uptake of [H]uridine was measured from an external solution containing 4 mM labeled uridine into cells containing varying concentrations of labeled uridine, as indicated. The external and internal concentrations of [H]uridine were set at equal specific activities. The net uptake of [H]uridine was measured by the stopper technique. Lines were obtained by linear regression.

**Unstirred Layers**

The infinite-cis procedure may also be used to evaluate the possibility that unstirred layers may play a role in transport. The unstirred layer has a retarding effect in those cases where fluxes are particularly high, thus causing clear deviations from the classical behavior of a simple carrier. On the basis of the magnitude of values related to initial
velocities of uridine fluxes it could be assumed that unstirred layers are likely not to affect the kinetic behavior of uridine transport. However, in order fully to elucidate this point it was decided to subject the system to a recently suggested test (9). The test relies on plotting infinite-cis data as \(1/v\) vs. \(S/(V - v)\), on the basis of the equation

\[
\frac{1}{v_{12}} = C^1 \frac{S_2}{V_{12} - v_{12}} - \frac{C^1}{P_2},
\]

where \(P_2\) is the effective permeability coefficient of the unstirred layer at side 2, \(C^1\) is a constant, and the other symbols have the meaning given by Lieb and Stein (9). Since the intercept on the \(S/(V - v)\) axis yields a value of \(1/P_2\) not significantly different from zero (Fig. 10) it can be concluded that unstirred layers need not be considered for uridine fluxes. Furthermore, the straight line obtained is indicative of results compatible with a simple carrier mechanism concerned with uridine transport.

**TABLE IV**

| Procedure      | Measured \(K^e\)  | Calculated \(K^e\) |
|----------------|-------------------|-------------------|
| \(ic - io\)    | 0.252±0.096       | 0.231             |
| \(ic - ei\)    | 0.987±0.226       | 1.08              |

\(K\) is given in mM.

**DISCUSSION**

In the present study the uridine transport system of human red blood cells is subjected to an extensive study of its kinetic properties. This study has relied on the application of several methodological and analytical approaches which constitute the basis for testing and characterizing a simple carrier mechanism (9). Previous application of these methods in conjunction with various rejection criteria has shed some serious doubts on the possibility that a simple carrier is
In the present work we attempt to demonstrate that the uridine transport responsible for the movement of hexoses in human red blood cells (2, 9). In fact, clear examples of simple carrier mechanisms operative in biological membranes are still to be provided (1).
system of red blood cells displays properties compatible with a simple asymmetric carrier. This claim is based on several lines of evidence.

First, it should be noted that an inherent property of the simple carrier requires the $V/K$ ratios for all experimental procedures to be equal. It can be seen from Table III that within the experimental errors the uridine transport system displays the aforementioned property. The variability found in the $V/K$ ratios stems primarily from the fact that measurements of $V$ are particularly sensitive to temperature. This is because many red blood cell transport systems, including the uridine carrier (unpublished observations), display a high activation energy (20–30 kcal/mol). Thus a slight deviation in temperature ($\pm 0.5^\circ C$) is likely to cause a significant change in the $V$ values ($K$ values are less susceptible to these deviations). Aside from the experimental errors related to the measurement of fluxes, these deviations contribute significantly to small differences in the calculated $V/K$ values. The importance of comparing the $V/K$ values for the different procedures while dealing with carrier systems, and especially those with asymmetric properties, has been implied in a recent review by LeFevre (2) in reference to the work of Baker and Widdas (16), Geck (17), and Wilbrandt (18), and by Regen and Tarpley (19).

Second, we proceed to test whether possible unstirred layer effects have to be considered in the analysis of uridine transport. This test was important in order to evaluate the possibility that the observed asymmetry of uridine transport stems from unstirred layer effects. We have demonstrated that unstirred layers need not be considered. In the case of the hexose transport systems of red blood cells, the asymmetric behavior of the carrier and some kinetic anomalies were attributed to something analogous to unstirred layer effects (19) although these effects were not tested directly.

Third, a method which can be used to test a simple carrier is based on predicting the kinetic parameters of infinite-cis ($K_{ci}^\infty$ and $K_{ci}^\infty$) from experimental kinetic parameters of exchange and zero-trans (both influx and efflux). If the experimental values do not coincide with the predicted values, then the simple carrier mechanism can be rejected. This test provides a means for evaluating the internal consistency of kinetic parameters obtained by the various experimental procedures.

We proceed to analyze the data according to the formalism of the one-complex model of a simple carrier (Fig. 11) as given by Lieb and Stein (9). The unidirectional flux from side 1 (inside) to side 2 (outside) is given by

$$V_{12} = \frac{KS_1 + S_2}{K^2R_{oo} + KR_{12}S_1 + KR_{21}S_2 + R_{ee}S_1S_2},$$

where $K$ is a basic measurable transport parameter, $S_1$ and $S_2$ are the respective intracellular and extracellular substrate concentrations. The $R$ symbols represent the basic measurable parameters related to the maximal velocities of transport:

$$R_{12} = R_{i0} = 1/V_{i0}^\infty; \quad R_{21} = R_{o1} = 1/V_{o1}^\infty; \quad R_{ee} = 1/V_{ee}^\infty;$$

and also

$$R_{i0} + R_{o1} = R_{ee} + R_{oo},$$
FIGURE 10 (above). Test for unstirred layer effects in uridine transport. The infinite-cis efflux experiment (●) tests the existence of unstirred layer effects on the outer side of the membrane while infinite-cis influx (○) examines this effect at the inner side. A positive intercept on the $S/(V_{\text{max}} - v)$ axis indicates the existence of unstirred layers. The values of $V_{\text{max}}$ taken for the calculation of $S/(V_{\text{max}} - v)$ were obtained from the least squares regression of $1/v$ against $S_i$, the cellular uridine concentration. The intercept on the abscissa for the efflux data is $0.03 \pm 0.32$ min$^{-1}$, i.e. not significantly different from zero. The intercept on the abscissa for influx is $-3.65 \pm 0.16$ min$^{-1}$, thus significantly different from zero. This rules out any possible effect of unstirred layers; however, since the intercept is negative, it could imply a cooperative phenomenon. No other evidence was found to support this view.

FIGURE 11 (below). The one-complex form of the simple carrier.

where $R_{\text{oo}}$ is the resistance to the movement of unloaded carriers. The experimental parameters in terms of $K$ and $R$'s are given in the Appendix (Table A I). We derive $R_{\text{oo}}$ from Eqs. (8) and (9) using the maximal velocities given in Table III. $R_{\text{oo}}$ is taken as the reciprocal of the mean of the slightly different values of $V_{\text{eff}}$ (Table III). Thus:

$$R_{\text{oo}} = R_{\text{io}} + R_{\text{ol}} - R_{\text{oe}}$$
$$= 0.403 + 1.883 - 0.133$$
$$= 2.255.$$

Using the calculated value of $R_{\text{oo}}$ in conjunction with several experimental
parameters we can estimate the Michaelis constants for the infinite-cis procedure, both efflux and influx.

These calculations are based on the following relationships:

\[ K_{\text{ee}}^{\text{c}} = \frac{K_{\text{ee}}^{\text{e}} \cdot R_{\text{ee}}^{\text{e}}}{R_{\text{ee}}^{\text{e}}} \tag{10} \]

and

\[ K_{\text{lo}}^{\text{c}} = \frac{K_{\text{lo}}^{\text{e}} \cdot R_{\text{lo}}^{\text{e}}}{R_{\text{ee}}^{\text{e}}} \tag{11} \]

The predicted \( K^c \) values computed from these relationships (Eqs. [10] and [11]) and those obtained experimentally are depicted in Table IV. It can be seen that both values are in accord with each other and are internally consistent with the kinetic properties that characterize the simple carrier mechanism.

Having shown that the uridine transport system has the properties of a simple carrier, we proceed to test this hypothesis further by applying additional rejection criteria (14). The first criterion is based on the relation

\[ K_{\text{ee}}^{\text{e}} K_{\text{lo}}^{\text{e}} = K_{\text{lo}}^{\text{e}} + K_{\text{ee}}^{\text{e}} \tag{12} \]

where \( Q \), the asymmetry factor, is given by

\[ Q = \frac{K_{\text{ee}}^{\text{e}}}{K_{\text{lo}}^{\text{e}}} = \frac{K_{\text{ee}}^{\text{e}}}{K_{\text{lo}}^{\text{e}}} = V_{\text{ee}}^{\text{e}} \tag{13} \]

Since the right-hand side of Eq. (12) gives 5.45 ± 2.73 as compared with \( Q + 1 = 5.5 \pm 0.58 \), the simple carrier cannot be rejected (\( Q \) was taken as the average value of the three different ratios of Eq. [13]). The second criterion is based on the above relation but transformed into a quadratic expression in \( K_{\text{ee}}^{\text{e}} \).

\[ \frac{(K_{\text{ee}}^{\text{e}})^2}{(K_{\text{lo}}^{\text{e}})} - (Q + 1) \cdot K_{\text{ee}}^{\text{e}} + K_{\text{lo}}^{\text{e}} = 0, \tag{14} \]

which yields

\[ Q + 1 \geq 2 \left( \frac{K_{\text{lo}}^{\text{e}}}{K_{\text{lo}}^{\text{e}}} \right)^{1/2}. \tag{15} \]

The right-hand side of this inequality is equal to 5.61, comparable to \( Q + 1 = 5.5 \).

The use of these criteria on the glucose system of human erythrocytes led Hankin et al. (14) to reject a simple carrier model. Thus the simple carrier model for the uridine transport system of human red blood cells cannot be rejected by the application of the various rejection criteria.

In conclusion, we show that the simple carrier model has satisfied all the tests to which it was subjected. There is therefore no need to postulate a more complex model for uridine transport in human erythrocytes. To the best of our knowledge we have here a unique example of an asymmetric simple carrier and hence one that can be used to test the adequacy of new methodologies of transport as well as internal consistency.
Several important features of the uridine carrier emerge from the present work. We recognize that the system displays a considerable degree of asymmetry as defined by the asymmetry factor $Q$. The value of the latter ranges from 3.5 to 5 displaying both a high $K^e$ and a high $V^e$ value at the inner side of the membrane. Although the difference in chemical affinities between the two sides of the membrane is compatible with the asymmetric properties of the cell membrane, it would seem premature to imply any structural counterpart to this property.

Proceeding with the inspection of the kinetic analysis of the simple carrier, we note that there are four independent parameters, i.e. $V^e$, $V^o$, $V^o^e$, and one Michaelis constant (either $K^e$, $K^o$, or $K^o^e$) which can be measured. However, there are five independent molecular rate constants, stemming from the following constraints

$$f_1b_1 = f_1b_2k_1,$$

(see Fig. 11). Therefore, unlike the case of a simple pore where the exact values of all possible ratios of rate constants can be computed, in the carrier we can obtain only the bounds for some ratios. Expressing the ratios of the $R$ parameters in terms of molecular rate constants (9; see also Appendix) we found that the upper and lower bounds for $k_1/k_2$ are given by:

$$\frac{R_{oo} - R_{ol}}{K_{ol}} < \frac{k_1}{k_2} < \frac{R_{io}}{R_{oo} - R_{lo}},$$

which in the present case is

$$0.198 < \frac{k_1}{k_2} < 0.288.$$

In the same way we could find the lower bound for $b_1/k_1$:

$$\frac{b_1}{k_1} > \frac{R_{ol} - R_{ee}}{R_{ee}} = 13.2,$$

and for $b_2/k_2$

$$\frac{b_2}{k_2} > \frac{R_{io} - R_{ee}}{R_{ee}} = 2.81.$$

According to the one-complex form of the carrier model (see Appendix), these inequalities imply that the asymmetry of the uridine transport system stems primarily from the fact that the rate constant for the movement of the unloaded carrier in the zero-trans influx condition is rate limiting. This is apparently true to a lesser degree in the efflux condition. The dissociation rates of the carrier-substrate complex are most probably not rate limiting inasmuch as $b_2 = 2.8k_2$ and $b_1 = 3.75k_2$ and since $k_2$ is itself greater than $k_1$. For hexoses, however, Wilbrandt (18) considered the translocation of the complex as not necessarily the rate-limiting step in the transport event. Since the present model is developed with the constraint that $f_1b_1k_2 = f_1b_2k_1$ and we have seen above that $0.2 < k_1/k_2 < 0.3$, then we learn that $f_1/b_2 = 4.3f_1/b_1$. Moreover, since $f_1/b_2$ represents the associa-
tion constant of the substrate-carrier complex, then the relative concentration of the complex at both sides of the membrane can be readily evaluated. Thus at equal concentrations of substrate on both sides of the membrane (i.e. conditions used to measure equilibrium exchange) the concentration of the complex is 3.5-5.1 times larger at the external surface of the membrane. Since there are apparently $10^4$ uridine carrier molecules per red blood cell membrane (20; Cabantchik and Hankin, unpublished observations) it would mean that the carrier molecules are distributed in such a manner that about 8,100 face the outer surface and 1,900 face the inner surface.

When one uses this figure of $10^4$ sites per cell, the calculated turnover rate for influx under exchange conditions at 25°C is about 7,600 molecules per carrier-min while for efflux it is about 35,000 molecules per carrier-min. Evidently, this finding awaits a more direct experimental confirmation. We should also mention that asymmetric properties are also observed in the hexose transport system of red blood cells (see reference 1 for review). However, in order to explain this phenomenon and the anomalous kinetic behavior of the system, authors such as Regen and Tarpley (19) invoked not only differential resistances of carrier movement but also a contribution of diffusion barriers. For uridine transport the latter contribution need not be considered.

It is particularly interesting to evaluate the present work in terms of current concepts of membrane structure. In principle the uridine carrier can function as a "mobile carrier" or as a protein with gating-type properties (21). Recent evidence suggests that a transmembrane protein with gating properties might be involved in anion transport in red blood cells (22). We note also that the carrier of anions in human red blood cells displays asymmetric properties in terms of susceptibility both to impermeant chemical modifiers and to agents such as phloridzin (23, 24). A similar chemical approach applied to the uridine system would provide some useful information for the understanding of the underlying molecular mechanisms of transport.

In summary, we have provided evidence which is compatible with the hypothesis that uridine movement across red blood cell membranes proceeds via a simple carrier mechanism which displays asymmetry properties. To our knowledge, this is a unique example of such a simple carrier system in the rather controversial carrier field.

APPENDIX

The results of the present work have been analyzed according to Lieb and Stein’s “Testing and characterizing the simple carrier” (9). The main relevant features of this analysis are summarized in Fig. 11, Eqs. (7)-(9), and this Appendix. The experimental parameters in terms of $K$ and $R$’s (Eq. [17]) are given in Table A I. The basic parameters can be expressed in terms of the rate constants $k, f$, and $b$ (see text and Fig. 11) as shown in Table A II. It is obvious from Table A II that there are four independent measurable parameters and five independent rate constants. For this reason the rate constants cannot be computed exactly, but it is possible to obtain bounds for the ratios of certain molecular constants. Bounds derived from Eqs. (25) and (26) and Table III of reference 9 are given in Table A III. As has been discussed by these authors, it is possible to compute both
upper and lower bounds only for one out of four ratios of rate constants related to a particular carrier. However, one bound, either upper or lower, can be obtained for two additional ratios as well.

**TABLE A1**

INTERPRETATION OF EXPERIMENTAL DATA IN TERMS OF BASIC MEASURABLE PARAMETERS

| Experimental procedure | 1. Net Flows  |
|------------------------|--------------|
| A. Zero-trans efflux   | \( \frac{V_{10}^e}{K_{10}^e} = \frac{1}{R_{12}^e} K_{12}^e = K \frac{R_{10}}{R_{12}} \) |
| influx                 | \( \frac{V_{10}^i}{K_{10}^i} = \frac{1}{R_{21}^i} K_{21}^i = K \frac{R_{10}}{R_{21}} \) |
| B. Infinite-cis efflux | \( \frac{V_{10}^e}{K_{10}^e} = \frac{1}{R_{12}^e} K_{12}^e = K \frac{R_{10}}{R_{12}} \) |
| influx                 | \( \frac{V_{10}^i}{K_{10}^i} = \frac{1}{R_{21}^i} K_{21}^i = K \frac{R_{10}}{R_{21}} \) |

2. Unidirectional fluxes: equilibrium exchange

\[
\frac{V_{10}^e}{K_{10}^e} = \frac{1}{R_{1e}} = \frac{1}{R_{2e}}, \quad \frac{K_{10}^e}{K_{20}^e} = K \frac{R_{10}}{R_{20}}
\]

**TABLE AII**

DEFINITION OF BASIC MOLECULAR PARAMETERS IN TERMS OF RATE CONSTANTS

\[
n_{R_{12}} = \frac{1}{b_2} + \frac{1}{k_2}, \quad K = \frac{b_k f}{b_1 + \frac{1}{f}} \left( \frac{1}{b_1} + \frac{1}{b_2} \right)
\]

\[
n_{R_{11}} = \frac{1}{b_1} + \frac{1}{k_1}, \quad \text{Constraint:} \quad f_2 b_1 k_1 = f_1 b_2 k_2
\]

\[
n_{R_{10}} = \frac{1}{k_1} + \frac{1}{k_2}
\]

\[
n_{R_{ee}} = \frac{1}{b_1} + \frac{1}{b_2}
\]

\( n \) is the total number of carriers per unit area of membrane while \( nR \) is the specific resistance of the membrane to the particular carrier-form.

**TABLE AIII**

UPPER AND LOWER BOUNDS FOR RATIOS OF RATE CONSTANTS IN TERMS OF BASIC MEASURABLE PARAMETERS

\[
\frac{R_{ee} - R_{10}}{R_{10}} < \frac{b_1}{b_2} < \frac{R_{10}}{R_{ee} - R_{10}}
\]

\[
\frac{R_{ee} - R_{10}}{R_{10}} < \frac{b_1}{b_2} < \frac{R_{10}}{R_{ee} - R_{10}}
\]

\[
\frac{R_{ee} - R_{10}}{R_{10}} < \frac{b_1}{b_2} < \frac{R_{ee} - R_{10}}{R_{ee} - R_{ee}}
\]

\[
\frac{R_{ee} - R_{10}}{R_{10}} < \frac{b_1}{b_2} < \frac{R_{ee} - R_{10}}{R_{ee} - R_{ee}}
\]
We thank Ms. S. Yeroushalmi and Ms. B. Hankin for their skillful technical assistance. We gratefully acknowledge Professor W. D. Stein’s comments, suggestions, and interest in this work. We thank Dr. Y. Eilam for critical reading of the manuscript.

This work was supported by grants to Z. Iov Cabantchik from the U. S. A.-Israel Binational Science Foundation and the Bat Sheba de Rothschild Foundation, and by National Cancer Institute contract no. 1 CP 43307 to W. D. Stein and Hagai Ginsburg. Dr. Cabantchik was a Lady Davis Fellow of the Hebrew University.

Received for publication 13 July 1976.

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