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L-Leucine Transport in Human Red Blood Cells: A Detailed Kinetic Analysis

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Summary. The kinetic properties of L-leucine transport across the human red blood cell membrane was analyzed according to the simple pore and carrier theory of Lieb and Stein (*Biochim. Biophys. Acta*, 1974, **373**:165–177 and 178–196) at 25 °C, pH 7.4. Several methods were used in order to obtain a thorough kinetic description of L-leucine transport. A rejection of the simple pore model was suggested from the results of zero-trans influx and zero-trans and equilibrium-exchange efflux experiments. Several predictions from the simple carrier model, based on the requirement of consistency among different kinetic parameters, were tested in infinite experiments, i.e. experiments performed at a high concentration of substrate at one of the faces of the membrane. The simple pore model was rejected, but no crucial evidence against a simple carrier model, which displays symmetric properties at 25 °C, was found in the concentration range considered (0.002–68 mM). The relative magnitudes of the rate constants of the translocation process are discussed, and it is concluded (a) that both the dissociation and translocation of carrier-complex is faster than the translocation of the empty carrier, (b) that no translocation step is rate determining, and (c) that the carrier-complex is equally distributed across the membrane at equilibrium. The present work provides a unique example of a carrier-mediated transport mechanism which displays symmetric properties. L-leucine transport in red blood cells may be a convenient system for studying molecular mechanisms of facilitated transport.

Key words: L-leucine transport, human red blood cell (erythrocyte), kinetic analysis, carrier model

The membrane of the human red blood cell (hRBC) has been the object of detailed investigations of transport phenomena, although until recently, the transport of amino acids has been a somewhat neglected field.

Five different transport systems have been described to account for the results of amino acid influx experiments on kinetics, substrate specificity and sodium dependence: an L-system for L-leucine, L-phenylalanine, L-methionine and L-valine (Winter & Christensen, 1964; Rosenberg & Rafaelsen, 1979; Young, Jones & Ellory, 1980), an Ly-system for L-lysine, L-ornithine and L-arginine (Gardner & Levy, 1972; Young et al., 1980), a T-system for L-tryptophan and L-tyrosine (Rosenberg, Young & Ellory, 1980), an ASC-system for L-alanine and L-cysteine (Young et al., 1980) and a glycine transporting system (Ellory, Jones & Young, 1980).

L-leucine transport has been characterized kinetically in some detail by Hoare (1972*a*, *b*). Data from equilibrium-exchange and net influx and efflux experiments were found consistent with carrier-mediated transport when fluxes were measured over a concentration range of 0.001–20 mM and over a temperature range of 0–37 °C (pH 7.4). The presence of a high-capacity, nonsaturable route, in addition to the carrier-mediated route, has been reported in studies by Winter and Christensen (1964) and by Hider and McCormack (1980). Young et al. (1980) were able to fit influx data to both a two- and three-parameter model, but they found that the nonsaturable route was definitely not of the magnitude previously found. The reasons for the discrepancy in experimental data from different groups are obscure, although some limitations of the studies are insufficient evidence that initial rate of unidirectional influx was obtained throughout the concentration range studied, and that statistical analysis for the goodness of fit by different kinetic models to the data have not been presented (*cf.* Atkins & Gardner, 1977).

New kinetic models have recently been described as candidates to supplant the classical carrier model

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of Wilbrandt and Rosenberg (1961), in order to account for observations of transport which have been difficult to explain by their model. Kinetic analysis of carrier-mediated transport based on relatively few assumptions concerning the translocation steps have been developed by Regen and Morgan (1964), Hoare (1972*a*), Lieb and Stein (1974*a*, *b*), Regen and Tarply (1974), and Devés and Krupka (1979). Although the simple carrier model of Hoare was consistent with the available kinetic data, an important question remained to be answered, namely, to what extent kinetic data can provide evidence for the validity of a carrier mechanism (Hoare, 1973). An essential aspect of the kinetic analysis of Lieb and Stein (1974*a*, *b*) is that a simple pore and a simple carrier model as well as different multiple carrier models (Eilam, 1977) can be subjected to a rigorous test of validity based on the requirement of consistency among kinetic parameters determined in different experimental procedures. Furthermore a kinetic criterion for the presence of separate transport systems is given in terms of the carrier model. In addition, analysis of voltage dependence of the kinetic parameters (Stein, 1977) and of irreversible inhibition of transport (Lieb & Stein, 1976) have been developed.

Considering the conflicting views on the adequacy of the carrier model to account for L-leucine transport in the hRBC, L-leucine transport was subjected to a rigorous analysis following the approach of Lieb and Stein (1974*a*, *b*). All experiments were performed at pH 7.4, and 25 °C, a temperature considered convenient to measure initial rates of fluxes. The nomenclature of the present paper is in accordance with Lieb and Stein (1974*a*, *b*).

Materials and Methods

L-leucine and HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) were obtained from Sigma Chemical Co., phloretin from K and K Laboratories, New York, DBP (di-*n*-butylphtalate (specific gravity 1.02–1.05)) from Merck and Co., L-(1-^{14}C)-leucine from New England Corp., L-(U-^{14}C)-leucine from Radiochemical Centre, Amersham, Instagel® from Packard Instruments. Other chemicals were of analytical grade.

Influx Experiments

Freshly dawn heparinized human blood from the same donor was washed 4–6 times with 10 volumes of isotonic buffered saline (in mM: NaCl 145, KCl 5, $MgCl_2$ 1, Na_2HPO_4 1, HEPES 15) at room temperature, discarding the buffy coat. The cells were equilibrated once for 15 min with 10 volumes of buffered saline to empty the cells of L-leucine. The cells were packed in nylon tubes by centrifugation at 0 °C in a refrigerated centrifuge (Sorvall 2RC-2B, Ivan Sorvall Inc.) for 20 min (35,000 × *g*) giving a trapped extracellular volume of less than 4% (vol/wt). In some experiments the cells were adjusted to a final hematocrit of about 20% in buffered saline. The cells were kept at 2 °C until shortly before the experiments. When used within 36 hr, no alteration of transport capacity of the cells could be demonstrated in control experiments (not shown).

Zero-trans Influx (zt_{12}) *Experiments.* In a test tube placed in a thermostat-jacket preheated red cells (25 °C) were transferred to 1–2 ml buffered saline with labeled L-leucine (generally about 1 μCi/ml) and vigorously stirred to give a hematocrit of 1–10%. The incubation was stopped at predetermined time intervals by transferring 100–200 μl incubate to 800 μl of stopping solution (ice-cold buffered saline with 250 μM phloretin) placed on top of 400 μl DBP in an Eppendorf test tube (3810). The cells were separated from the incubation medium by centrifugation in an Eppendorf Microcentrifuge (model 3220). The cells were washed once with 1000 μl of stopping solution (0 °C) and hemolyzed by adding 500 μl saponine (10 mg%). 100 μl 27% (vol/vol) trichloroacetic acid was added and after centrifugation, 500 μl supernatant was transferred to 10 ml Instagel® for counting in a Packard Instrument Scintillation Spectrometer (model 2425). Counts were corrected for quenching by internal and external standards, the correction factor being at maximum 1.1. The mean recovery of red cells was 90%, and the apparent cellular activity was corrected for recovery and sometimes also for the amount of extracellular medium carried over through the washing procedure (mean value: 0.7% of the activity within the cells at equilibrium).

For the study of transport it is necessary to measure the intracellular concentrations after precisely determined time intervals. The transport process was therefore stopped instantaneously by the combined effects of an abrupt lowering of the rate of transport by lowering the temperature, the dilution of the incubate with stopping solution and the presence of 250 μM phloretin in the stopping solution.

Infinite-trans Influx (it_{12}) *Experiments* were performed similarly except that cells were loaded with unlabeled L-leucine (80 mM in D-glucose (10 mM) containing saline, hematocrit 30–40% for 3 to $3\frac{1}{2}$ hr at 37 °C) to give "infinitely" high concentrations (Stein & Lieb, 1973) of L-leucine within the cells. The intracellular concentrations obtained during the loading procedure were measured in cells that had been loaded with both labeled and unlabeled L-leucine. 100 μg of the labeled cells were packed in nylon tubes, hemolyzed, precipitated by trichloroacetic acid and then centrifuged. The cells loaded with L-leucine to be used for the infinite experiments were always packed in nylon tubes by centrifugation to minimize the amount of trapped medium between the cells. In the it_{12} experiments the hematocrit was $\frac{1}{2}$ to 1%. The contribution to the extracellular concentration in influx experiments of the amino acid in the trapped volume was about 15 μM. Small corrections were needed, therefore, only at the lowest concentrations used. Control experiments showed that at 43 mM, the permeability for L-leucine measured in zt_{21} experiments was found to be unaffected by prior loading of the cells with 80 mM L-leucine media, both where iso-osmolality was or was not maintained by adjusting the incubation medium NaCl concentration (data not shown).

Efflux Experiments

Zero-trans (zt_{21}) *and Equilibrium-Exchange* (ee_{21}) *Efflux Experiments.* The concentration of L-leucine was measured in cells loaded at a given specific activity, and cells packed in nylon tubes were injected into 15–50 ml of the vigorously stirred medium (either isotonic buffered saline without L-leucine or with L-leucine at the same concentration as within the cells). The cells were separated from the medium at predetermined time intervals by

the rapid filtration method of Dalmark and Wieth (1972) or by centrifugation in an Eppendorf microcentrifuge 3220 followed by immediate removal of part of the supernatant. Counting was performed as described above. The two methods gave identical results, but a smaller amount of medium was needed for the last method, which was used most often. In some efflux experiments the concentration of the labeled compound was determined within the cells, which were then separated from the medium by centrifugation through DBP, and treated as described above, but generally part of the incubation medium was transferred for counting. Hematocrits in efflux experiments were $\frac{1}{2}$ to 2%. The concentration at equilibrium was obtained by precipitation of a sample of the incubate with trichloroacetic acid. Control experiments showed that identical concentrations were measured when the efflux of tracer was followed to equilibrium or when a sample of incubate was precipitated.

Infinite-cis Efflux (ic_{21}) Experiments. Cells were loaded with an "infinitely" high concentration of L-leucine at a given specific activity, packed in nylon tubes and the intracellular concentration was determined. The rate of net efflux was measured at different extracellular concentrations of L-leucine that had the same specific activities as the L-leucine within the cells. The hematocrit was $\frac{1}{2}$ to 1%. The concentration of tracer was measured within the cells as a function of time after separation of the cells from the medium by centrifugation through DBP in suitable time intervals.

Determination of Cell Volume and Water Content

The number and the volume distribution of the cells was determined using a Coulter Counter (model DN, Coulter Electronics Inc., Hialeah, Fla.). The percentage of water per wet weight of the cells (0.64 wt/wt) was determined by drying the cells to a constant weight (105 °C, 24 hr). The ratio v/A [cell water volume/area (=volume-independent membrane area)] was determined as a function of the osmolality of the buffered saline, assuming a membrane area of 1.42×10^{-6} cm^2 (Funder, Tosteson & Wieth, 1978):

$$v/A = (-9.24\,p + 6.81) \times 10^{-5}\ (\text{cm})$$

where p is osmol/liter.

Calculation of Concentration

The intracellular activity at time t $a(t)$ (dpm/liter cell water) was calculated from the equation:

$$a(t) = D \cdot I \cdot K \cdot E^{-1}$$

where D is the activity (dpm) of the sample corrected for background, quenching, recovery and trapped medium, I the absorbance of one liter of cells at 300 mosm/liter, E the absorbance of the sample and K a normalization constant.

$a(t)$ is then equal to dpm/cell unit, a cell unit being that number of cells whose solvent water is one liter under isotonic condition. The concentration (mmol/liter cell water) was then calculated from the specific activity (dpm/mmol) and a calibration curve relating the water content of the cells to the osmolarity of the medium. The calibration factor was at maximum 15%.

When cells were loaded for flux experiments, the cellular activity was measured similarly, except that the amount of cells was obtained weighing.

Calculation of the Initial Rate of the Unidirectional Flux

The net flux $j_{12}(t)$ (mmol/(cm^2 × sec)) of L-leucine through the membrane at time t in the direction (1) → (2) is given by

$$j_{12}(t) = P(S_2 - S_1)$$

where P is the permeability (cm/sec) for L-leucine, and S_1 and S_2 the concentration (mmol/cm^3) of L-leucine in the medium and in the cells, respectively (Sten-Knudsen, 1978).

At equilibrium, identical concentrations of L-leucine were measured in the medium and in the cells (concentration range: 0.02–67.6 mM). Assuming that isotope effect can be neglected and that the profile of substrate concentration in the membrane can be treated as a quasi-stationary diffusion process, ^{14}C-L-leucine transport across the membrane can be analyzed by applying a closed two-compartment model with a single value for the rate coefficient k (sec^{-1}) of tracer exchange. Thus,

$$a(t) = (a_\infty - a_0)(1 - \exp(-kt)) + a_0$$

where $a(t)$ is the activity (dpm/liter) in the cells (influx experiments) or in the medium (efflux experiments) at time t of sampling, a_0 (dpm/liter) and a_∞ (dpm/liter) the tracer concentration at time zero in the medium (caused by trapped extracellular medium between the cells) and at equilibrium (*cf.* Sten-Knudsen, 1978). If the rate coefficient is estimated by linear least-square regression of $\ln(1 - a(t)/a_\infty)$ *vs.* t, the intercept on the ordinare is equal to $\ln(1 - a_0/a_\infty)$. The rate coefficients were generally estimated from duplicates, each run consisting of three to five times of sampling. The standard error of the mean was typically less than 7%.

The *permeability* P (cm/sec) for L-leucine is then given by

$$k = P \cdot A(1/v_i + 1/v_e)$$

where v_i and v_e is the volume (cm^3) of the intra- and extracellular compartment, and A is the area (cm^2) of the membrane (Sten-Knudsen, 1978).

The *unidirectional flux* u_{ab} (mmol/(cm^2 × sec)) at the initial rate, to be used for the kinetic analysis, is given by

$$u_{ab} = P \cdot S_a^0$$

where S_a^0 is the initial concentration of L-leucine in compartment a. In zero-trans influx and efflux experiments it is important to use only the initial part of the time course curves as shown in the results section.

When efflux curves from ee_{21} experiments were followed for several half-times, a deviation from the expected monoexponential course was found. As log-normal distributions are commonly found in biological systems (Gaddum, 1945), a curve predicted from an ln-normal distribution of the rate constants of red blood cells was fitted to data from ee_{21} experiments at 0.5 mM L-leucine (Fig. 1) according to the equation

$$\varphi = (2\pi\lambda^2)^{-\frac{1}{2}} \int_{-\infty}^{\infty} \exp(-kt - (\ln k - \overline{\ln k})^2/2\lambda^2)\, d \ln k$$

(Creese, Neil & Stephenson, 1956) where φ is the fraction of the activity remaining in the cells, and where the rate constant k is distributed from $k=0$ to $k=\infty$ with geometric mean k_m such that $\overline{\ln k} = \ln k_m \cdot \lambda$ is the standard deviation of the Napierian logarithms and t time.

From visual fits of predicted curves as given by Creese et al. (1956) to the experimental data, λ (in log unit) was estimated within the range 0.13–0.22. A probit analysis of the volume distribution of red cells gave a standard deviation of about 0.15 (log unit) (not shown), suggesting that the heterogeneity of the rate constants may be partly due to a variation of the water content of the cells, if the membrane area is assumed to be independent of volume.

The efflux curve did not deviate much from a monoexponential decrease, when the efflux was followed for less than 1 half-time. The time taken for the cellular activity to fall to e^{-1} of the initial value gives the geometric mean time constant k_m^{-1}. As t

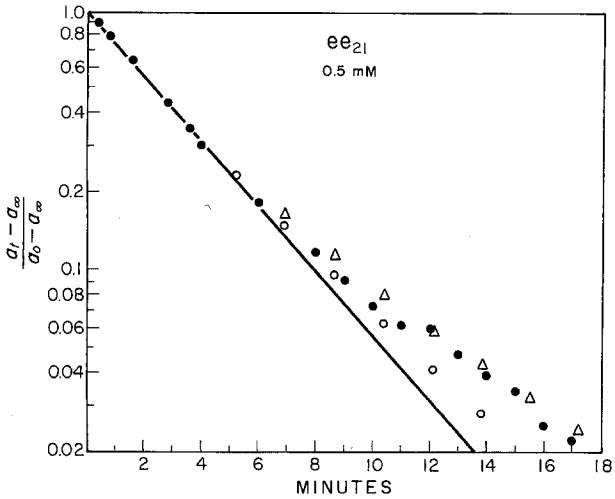

Fig. 1. Equilibrium-exchange efflux (ee_{21}) experiment in human red blood cells at 0.5 mM L-leucine (25 °C, pH 7.4, hematocrit: 1 %). *Ordinate* (log-axis): $(a(t)-a_\infty)/(a_0-a_\infty)$, where a_0, $a(t)$ and a_∞ is the cellular concentration of ^{14}C-L-leucine at time zero, t and at equilibrium. *Abscissa:* time in minutes. Experimental data are indicated by (●) and predicted data assuming a ln-normal distribution of the rate constants of exchange by (○) (standard deviation: $\lambda=0.13$ (log-unit) or by (△) ($\lambda=0.22$)). *See* Materials and Methods for further explanation

approaches zero, the rate coefficient approaches the arithmetic mean constant $\bar{k}$, which provides a consistent estimate of the population mean (Finney, 1941). $\bar{k}$ and k_m is related by $\bar{k}=k_m \exp(\lambda^2/2)$ (Creese et al., 1956). Thus by assuming a monoexponential decrease of the cellular activity the regression estimate of the rate coefficient k will be only somewhat (by 10% or less) smaller than the arithmetic mean constant $\bar{k}$ estimated by assuming heterogeneity among the rate coefficients of the cells. It was therefore considered justified to use a two-compartment model with a single value of the rate coefficient k and the volume of the cellular compartment v_i.

In *infinite-trans influx experiments*, uptake curves were linear for much longer periods of time in zt_{12} experiments (*see* Results). The unidirectional flux at the initial rate could thus simply be estimated by linear least-square regression of dpm/(liter · a_s) against time, where dpm/liter is the concentration of tracer within the cell and a_s, the initial specific activity (dpm/mmol) of L-leucine.

When in *infinite-cis efflux* the concentration of tracer (dpm/liter) was measured in the cells as a function of time, the relationship was linear during the period of sampling and independent of the given extracellular concentration of L-leucine. The rate of net efflux (mmol/(liter × min)) was estimated by linear regression of dpm/liter *vs.* t. The intercept on the ordinate corrected for trapped extracellular medium is equal to the initial concentration of tracer. The fractional initial rate of tracer efflux k (min^{-1}) was then obtained from the ratio of the slope and the intercept on the ordinate of the regression line. The initial rate of efflux u_{21} (mmol)/(liter × min) to be used for the kinetic analysis is then given by

$$u_{21}=k \cdot S_2^0$$

where S_2^0 is the initial concentration of L-leucine within the cells.

Statistical Analysis

Linear least-square regression analysis was performed by a Hewlett-Packard (9810A) calculator. Nonlinear least-square regression analysis using a Gauss-Newton algorithm (Jennrich, 1977) was performed by an IBM 370/165 computer. Data were fitted to a two- and a three-parameter model:

$$u=V \cdot S \cdot (K+S)^{-1}$$

$$u=V \cdot S \cdot (K+S)^{-1}+K_D \cdot S$$

where u is the initial rate of the unidirectional flux (mmol/(liter × min)), V the maximum velocity (mmol/(liter × min)), K the half-maximum velocity concentration constant (mM), K_D (min^{-1}) the rate coefficient of a nonsaturable component in parallel with the Michaelis-Menten component, and S the concentration of L-leucine (mM). Before fitting of the data the fluxes (original unit: mmol/(cm^2 × sec)) were converted to the unit of mmol/(liter × min).

The latter unit was preferred in the present study as it has been used in previous studies on amino acid transport in red blood cells.

The variances of various ratios and mean values of kinetic parameters constituting the testing and characterization of the simple pore and carrier models of Lieb and Stein (1974*a*, *b*) were estimated as suggested by Cleland (1967, Eq. 18).

Results

Equilibrium-Exchange (ee_{21}) and Zero-trans (zt_{21}) Efflux Experiment

Typical ee_{21} efflux curves at different concentrations of L-leucine are presented in Fig. 2*a*. The unidirectional flux v_{21}^{ee} (mmol × liter^{-1} × min^{-1}) as a function of L-leucine concentration is shown in Fig. 3.

In zt_{21} efflux experiments the extracellular concentration of L-leucine should be the zero, while remaining constant in the cells to obtain initial rates of efflux at a given cellular concentration of substrate. This was approximated by sampling in short time intervals from suspensions incubated at low hematocrits ($\frac{1}{2}$ to 1%). Typical efflux curves are presented in Fig. 2*b*.

At each of the concentrations studied (0.03–42.7 mM), linear least-square regression analysis of the first four or five data points *vs.* time yielded very high correlation coefficients ($r \geqq 0.99$). The unidirectional efflux at the initial rate v_{21}^{zt} was therefore calculated from the estimated rate coefficient of transport. A plot of v_{21}^{zt} *vs.* concentration of L-leucine is shown in Fig. 3.

At low concentrations of L-leucine (<0.5 mM) the fluxes were of similar magnitude in both kinds of efflux experiments, but at concentrations higher than about 5 mM, the efflux was apparently stimulated by the presence of substrate in the extracellular medium.

Zero-trans Influx (zt_{12}) Experiments

The flux *vs.* concentration was determined over a large concentration range (0.002–50 mM). The initial part of typical influx curves at four different con-

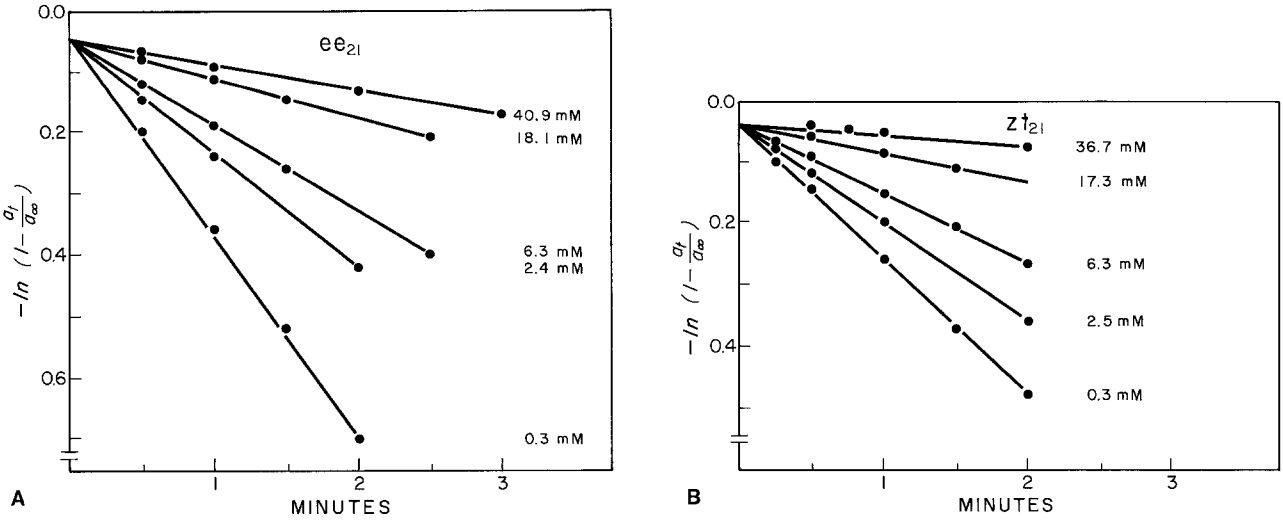

Fig. 2. Efflux experiments in human red blood cells (25 °C, pH 7.4, hematocrit: $\frac{1}{2}$–2 %) at different cellular concentrations of L-leucine (0.3–40.9 mM). *(a)* equilibrium-exchange efflux (ee_{21}) experiments. *(b)* zero-trans efflux (zt_{21}) experiments. *Ordinate:* $-\ln(1-a(t)/a_\infty)$, where $a(t)$ and a_∞ is the concentration of ^{14}C-leucine in the medium at time t and at equilibrium. *Abscissa:* time in minutes

centrations of L-leucine (0.05, 2.5, 10, 50 mM) are presented in Fig. 4. At low concentrations (0.1–0.2 mM) the curves were linear for at least two half-times of transport, while at higher concentrations (>5 mM) the slope of the curve quickly deviated from the initial value, which was to be used for estimation of the unidirectional influx at the initial rate. Linear regression of the first four data points generally gave regression coefficients ≧0.99. The unidirectional influx at the initial rate v_{12}^{zt}, as a function of L-leucine concentration, is shown in Fig. 5.

Test of Kinetic Models and Estimation of Kinetic Parameters

The means of the unidirectional fluxes at the initial rate (duplicate runs measured at 18–25 different concentrations of L-leucine within the range 0.3–50 mM) from zero-trans and equilibrium-exchange experiments were fitted to a two- and a three-parameter model by nonlinear least-square regression analysis (Table 1). Several kinds of evidence were considered when evaluating the goodness of fit of kinetic models (*cf.* Atkins, 1976; Atkins & Gardner, 1977; Table 2).

The final convergence to a regression solution was obtained after 2 to 3 steps of iteration by both models. The residual mean squares were not significantly reduced by augmenting the number of parameters when tested by an F-test (Draper & Smith, 1966; Vinten, Gliemann & Østerlind, 1976). The residuals plotted on probability paper appeared to be normally distributed, the serial correlation coefficients (Jennrich, 1977) were of moderate size and

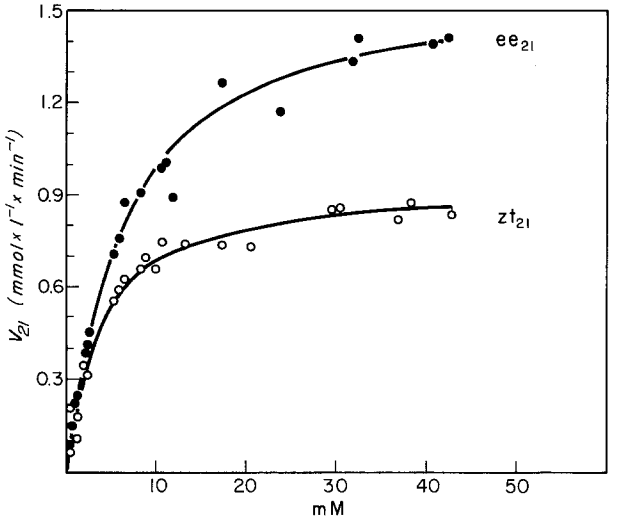

Fig. 3. Initial rate of unidirectional efflux (v_{21}) as a function of the cellular concentration of L-leucine from equilibrium-exchange efflux (ee_{21}) and zero-trans efflux (zt_{21}) experiments in human red blood cells (25 °C, pH 7.4, hematocrit: 1–3 %). *Ordinate:* mmol × liter^{-1} × min^{-1}. *Abscissa:* mM. Estimated kinetic parameters from efflux experiments are presented in Table 1

significant values were not obtained when examining runs of residuals in a sequence plot (Draper & Smith, 1966) when considering both kinetic models. As the variances of the fluxes were proportional to the fluxes themselves, when the reciprocal of fluxes squared were weighted according to Cleland (1967), no improvement of the fits were obtained (not shown). No definite evidence against a simple Michaelis-Menten relationship between flux and concentration was demonstrated in any of the experimental procedures studied, nor was the goodness of

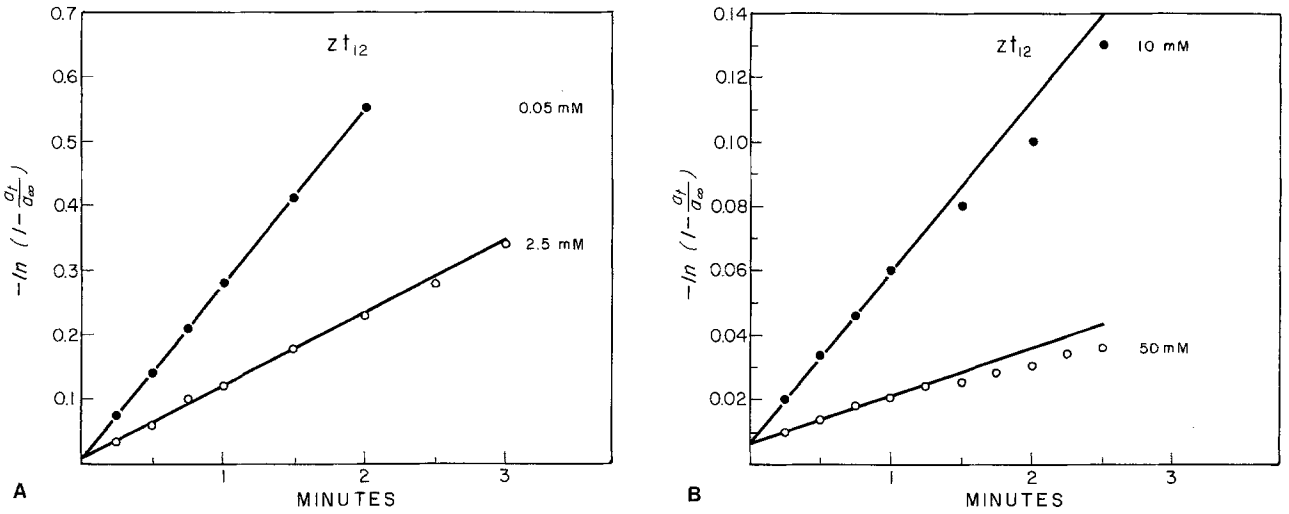

Fig. 4. Zero-trans influx (zt_{12}) experiments in human red blood cells at different concentrations of L-leucine (mM) (25 °C, pH 7.4, hematocrit: 5–10 %). *Ordinate* $-\ln(1-a(t)/a_\infty)$, where $a(t)$ and a_∞ is the concentration of ^{14}C-L-leucine at time t and at equilibrium. *Abscissa:* time in minutes. Note the different units of the ordinates in *(a)* and *(b)*

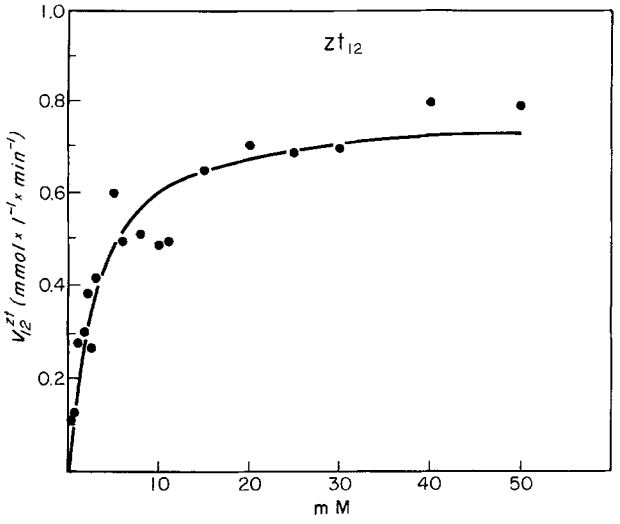

Fig. 5. Initial rate of the unidirectional influx (v_{12}^{zt}) as a function of the extracellular concentration of L-leucine from zero-trans influx experiments in human red blood cells (25 °C, pH 7.4). *Ordinate:* mmol × liter^{-1} × min^{-1}. *Abscissa:* mM. Estimated kinetic parameters are presented in Table 1

fit significantly improved when the three-parameter model was considered.

The validity of the kinetic models was further examined at low concentrations of L-leucine ($S \ll K_{\frac{1}{2}}$), where for all experimental procedures considered, the fluxes are predicted to be linearly related to the substrate concentration with slopes of V_{21}^{ee}/K_{21}^{ee}, V_{21}^{zt}/K_{21}^{zt} and V_{12}^{zt}/K_{12}^{zt} (two-parameter model). Experimentally determined and predicted values of the slopes are compared in Table 3 for both the two- and three-parameter model. Identical slope values were measured in all types of experiments, and were found to be the predicted magnitudes.

Data from zt_{12} and ee_{12} experiments were finally plotted as log (flux) *vs.* concentration as suggested by Hoare (1972*a*) to test for deviations from a Michaelis-Menten relationship. For the Michaelis-Menten case a slope of 1 is predicted (Fig. 6). In both cases curves with slopes not significantly exceeding 1.0 were obtained.

The independence of the kinetic parameters of time was studied in zt_{12} experiments. Mean values of the maximum velocity V_{12}^{zt} (±SEM) and of K_{12}^{zt} (±SEM) from five series of experiments performed within a period of 18 months with red cells from the same donor were V_{12}^{zt}: 0.691 ± 0.065 mmol × liter^{-1} × min^{-1} and K_{12}^{zt}: 2.91 ± 0.24 mM. This suggests that the parameters are time-independent characteristics of membrane transport.

Infinite-trans Influx (it_{12}) Experiments

The time course of the cellular concentration of tracer was determined at 0.4 and 53.5 mM L-leucine at time zero in the extra- and intracellular compartment (Fig. 7). A transient accumulation of tracer reaching a level of 7.7 times the concentration at equilibrium was measured. In zt_{12} experiments, concentrations above the equilibrium level were never found. The transient accumulation of tracer apparently driven by the gradient of unlabeled substrate is known as the countertransport phenomenon. The first part of the time course curves (cellular concentration of tracer plotted against time) from it_{12} experiments was linear for a much longer period

Table 1. Estimation of kinetic parameters by nonlinear least-square regression analysis (Jennrich, 1977) of data from equilibrium-exchange efflux (ee_{21}), zero-trans efflux (zt_{21}) and zero-trans influx (zt_{12}) experiments of L-leucine transport in human red blood cells (25 °C, pH 7.4)[a]

Experimental procedure	Number of parameters	V (mmol × liter^{-1} × min^{-1})	K (mM)	K_D (min^{-1})	Concentration range (mM)
ee_{21}	2	1.613 ± 0.041	6.56 ± 0.50	–	0.30 – 42.5 (22)
	3	1.464 ± 0.154	5.68 ± 0.99	0.0030 ± 0.0031	
zt_{21}	2=3	0.922 ± 0.015	3.45 ± 0.21	–	0.30 – 42.7 (25)
zt_{12}	2	0.771 ± 0.041	2.78 ± 0.58	–	0.35 – 50.0 (18)
	3	0.604 ± 0.072	1.60 ± 0.56	0.0046 ± 0.0020	

[a] A two parameter model ($v = V \times S/(K+S)$) and a three parameter model ($v = V \times S/(K+S) + K_D \times S$) were considered, where V is the maximum velocity, K the half-maximum constant, K_D the rate coefficient of a nonsaturable component, units as given in the Table. The experiments were performed as described in the text. Asymptotic standard deviations of kinetic parameters are shown in the Table. A mean flux from duplicates was measured at (n) different concentrations of L-leucine in the range indicated. Data from 4 or 5 series of experiments were pooled. Note that the same regression solution was obtained when the two kinetic models considered were fitted to data from zt_{21} experiments.

Table 2. Analysis of the goodness of fit of different regression solutions presented in Table 1[a]

Experimental procedure	Number of parameters	*RSS* × 10^{-2}	f	Convergence	*F*-test	Probit analysis	Serial correlation coefficient	Test of runs
ee_{21}	2	5.36	20	3	–	+ +	−0.42	0.14
	3	5.14	19	3	0.81[§]	+ +	−0.50	0.27
zt_{21}	2=3	1.94	23	3	–	+ + +	−0.18	0.55
zt_{12}	2	8.88	16	3	–	+ + +	0.18	0.41
	3	6.84	15	2	4.47[§]	+ + +	−0.03	0.55

[a] The following kind of evidence was considered: *Convergence:* number of iterations to obtain the final regression solution (tolerance for convergence 1×10^{-5}; *RSS*: residual sum of squares; f: degrees of freedom). *F-test:* test of reduction of the *RSS* by increasing the number of parameters of the fitted model as described by Draper and Smith (1966) and Vinten, Gliemann and Østerlind (1976). Values of *F*-distribution (degrees of freedom $1, f$) are given in the Table; §): $p > 0.05$. Further analysis of residuals comprised graphical *probit analysis:* (+ → + + + + +), a ranking scale indicating that residuals appeared normally distributed, calculation of a *serial correlation coefficient* as described by Jennrich (1977), and examining *runs* in a sequence plot as described by Draper and Smith (1966). Values in the Table give the probability level that the observed number for any given sequence of signs will occur. *See text* for further explanation.

Table 3. Comparison of the ratio of experimentally determined kinetic parameters (V/K min^{-1}) from zero-trans influx (zt_{12}), zero-trans efflux (zt_{21}) and equilibrium-exchange efflux (ee_{21}) experiments presented in Table 1 (two-parameter model) and the fractional rate coefficient of transport k (min^{-1}) measured at low concentrations of L-leucine (9–120 μM) relative to the concentration range applied for estimation of the kinetic parameters

Experimental procedure	V/K (min^{-1}) two-parameter model	$V/K + K_D$ (min^{-1}) three-parameter model	k (min^{-1})	Concentration range (μM)
ee_{21}	0.246 ± 0.019	0.260 ± 0.020	0.247 ± 0.007	30–120 (10)
zt_{21}	0.267 ± 0.017	–	0.262 ± 0.024	30–75 (10)
zt_{12}	0.277 ± 0.060	0.383 ± 0.095	0.263 ± 0.010	9–70 (8)

[a] k was determined at a hematocrit of 1 %. Considering a three-parameter model k (min^{-1}) and $V/K + K_D$ (K_D, the proportional constant of the linear component) were compared. The standard deviation of V/K was calculated according to Cleland (1967). The standard error of the mean rate coefficient k was determined from the number of experiments given in brackets. The predicted and measured rate coefficients were equal independent of the experimental procedure performed and the kinetic model considered. *See text* for further explanation.

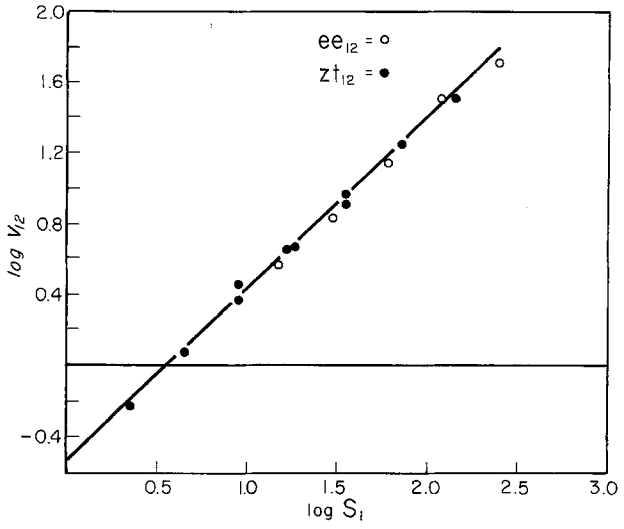

Fig. 6. Initial rate of unidirectional influx (v_{12}) as a function of the extracellular concentration of L-leucine from equilibrium-exchange influx (ee_{12}) and zero-trans influx (zt_{12}) experiments in human red blood cells (25 °C, pH 7.4). *Ordinate:* $\log v_{12}$. *Abscissa:* $\log S_1$, where S_1 is the concentration of L-leucine (range: 2–250 μM). Linear regression analysis yielded: $\log v_{12}^{zt} = 0.96 \times \log S_1 - 0.53$ ($r > 0.99$) and $\log v_{12}^{ee} = 0.97 \times \log S_1 - 0.57$ ($r > 0.99$), and no second order kinetics was thus indicated. *See text* for further explanation

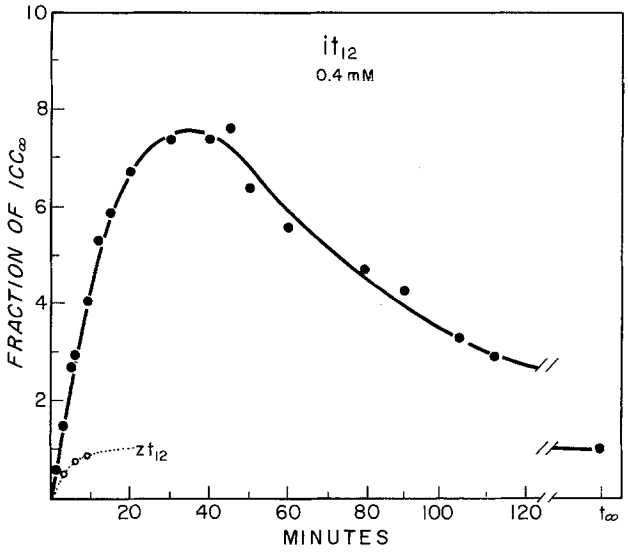

Fig. 7. Infinite-trans influx (it_{12}) experiment in human red blood cells (25 °C, pH 7.4, hematocrit: 0.7 %) at initial extra- and intracellular concentrations of 0.4 and 53.5 mM L-leucine. *Ordinate:* fraction of the concentration of ^{14}C-L-leucine at equilibrium. *Abscissa:* time in minutes. t_∞ indicates the time of equilibrium. Note the transient accumulation of ^{14}C-L-leucine. For comparison, a zero-trans influx (zt_{12}) experiment at 0.4 mM L-leucine is shown. *See text* for further explanation

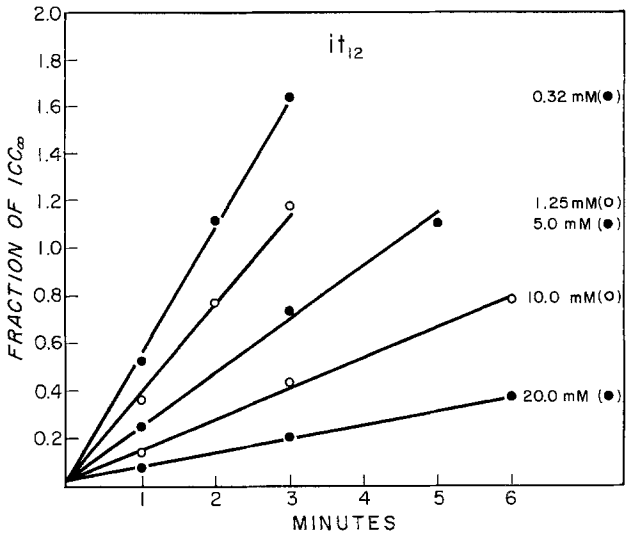

Fig. 8. Infinite-trans influx (it_{12}) experiments in human red blood cells at different extracellular concentrations of L-leucine (0.32–20 mM) (25 °C, pH 7.4, hematocrit: 0.5–1 %). Initial intracellular concentration: 55.9 mM. *Ordinate:* fraction of intracellular concentration of ^{14}C-L-leucine at equilibrium. *Abscissa:* time in minutes

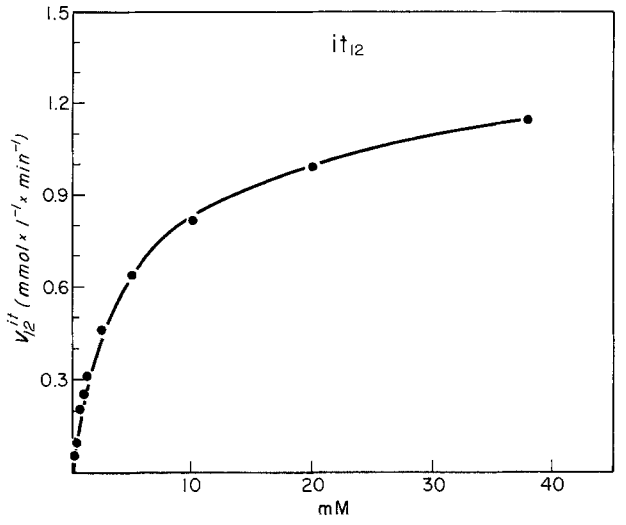

Fig. 9. Initial rate of the unidirectional influx (v_{12}^{it}) as a function of the extracellular concentration of L-leucine from infinite-trans influx (it_{12}) experiments in human red blood cells (25 °C, pH 7.4). *Ordinate:* mmol × liter^{-1} × min^{-1}. *Abscissa:* mM. Estimated kinetic parameters of it_{12} experiments are presented in Table 4

of time than in zt_{12} experiments (*cf.* Figs. 7 and 8). Furthermore, cellular concentrations above the equilibrium level were measured throughout the concentration range studied (0.3 to 20 mM), and at low concentrations of L-leucine ($S \ll K_{\frac{1}{2}}$) v_{12}^{it} was faster than the corresponding v_{12}^{zt} (*cf.* Fig. 7).

The initial rate of the unidirectional flux v_{12}^{it} (mmol × liter^{-1} × min^{-1}) as a function of L-leucine concentration is shown in Fig. 9. Results from non-linear least-square regression analysis of data from

Table 4. Estimation of kinetic parameters

Experiment	Concentration range (mM)	n	Regression analysis	V (mmol × liter^{-1} × min^{-1}) (±SD)	K (mM) (±SD)	Cellular concentration (mM)
it_{12}	0.16–38	10	nonlinear	1.219±0.035	4.24±0.38	40.1
	0.25–20	8	nonlinear	1.213±0.154	3.75±1.31	55.9
	0.25–20	8	nonlinear	1.415±0.112	3.09±0.71	53.5
	Mean±SEM			1.282±0.066	3.69±0.33	
ic_{21}	0–8.9	7	linear	0.946±0.140	1.75±0.33	67.6
	0–10	10	linear	0.797±0.103	2.38±0.43	54.6
	0.3–10	9	linear	1.032±0.226	1.81±0.49	64.8
	mean±SEM			0.925±0.069	1.98±0.20	

[a] V (maximum velocity: mmol × liter^{-1} × min^{-1}) and K (half-maximum constant: mM) from data of infinite-trans (it_{12}) and infinite-cis efflux (ic_{21}) experiments in human red blood cells (25 °C, pH 7.4) performed as described in Materials and Methods, by nonlinear regression analysis (it_{12} experiments) or by linear regression analysis of data from ic_{21} experiments transformed as shown in Fig. 11 followed by calculation of V_{21}^{ic} as described in Materials and Methods. n, the number of different concentrations of L-leucine in a series of experiments at a given intracellular concentration, is shown in the last column. All linear regression coefficients were ≧0.99. SD = standard deviation, SEM = standard error of the mean.

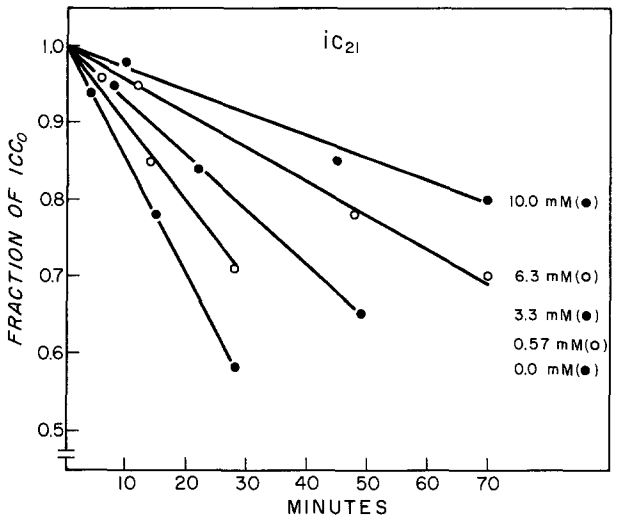

Fig. 10. Infinite-cis efflux (ic_{21}) experiments in human red blood cells at different extracellular concentrations of L-leucine (0–10.0 mM) (25 °C, pH 7.4, hematocrit: 0.5–1 %). Initial concentration within the cells: 54.6 mM. *Ordinate:* fraction of the concentration of ^{14}C-L-leucine within the cells at time zero. *Abscissa:* time in minutes

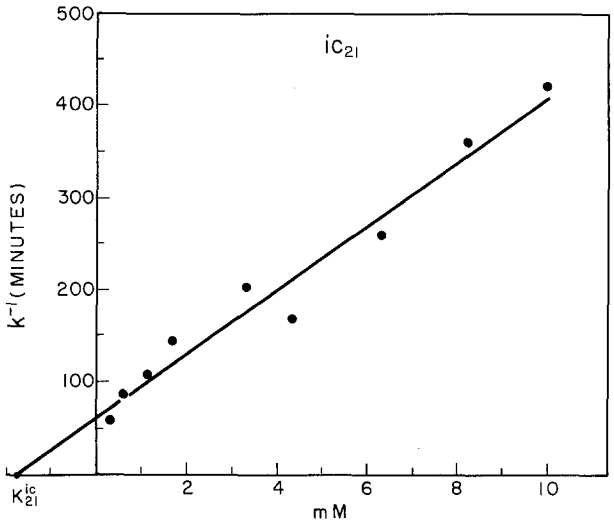

Fig. 11. Reciprocal of the initial fractional rate constant k (min^{-1}) from infinite-cis efflux (ic_{21}) experiments in human red blood cells as a function of the extracellular concentration of L-leucine (0.3–10.0 mM) (25 °C, pH 7.4). *Ordinate:* minutes. *Abscissa:* mM. The intercept on the ordinate is equal to the reciprocal of the maximum fractional rate constant k_0 (min^{-1}). The intercept on the abscissa is the extracellular L-leucine concentration of a fractional rate coefficient equal to $\frac{1}{2}k_0$. This concentration is defined as the kinetic parameter K_{21}^{ic} (mM). The maximum velocity V_{21}^{ic} (mmol × liter^{-1} × min^{-1}) is equal to $k_0 \times S_2$, where S_2 is the initial cellular concentration of L-leucine (64.8 mM). Estimated kinetic parameters from ic_{21} experiments are presented in Table 4

three series of experiments considering a two-parameter model are presented in Table 4. Mean values (±SEM) were $V_{12}^{it} = 1.282 \pm 0.066$ mmol × liter^{-1} × min^{-1} and $K_{12}^{it} = 3.69 \pm 0.33$ mM.

Infinite-cis Efflux (ic_{21}) Experiments

The rate of net efflux of L-leucine from cells loaded to a high cellular concentration of substrate (55 mM) at different extracellular concentrations of L-leucine is shown in Fig. 10. The curves were linear for much longer periods of time compared with the previously studied experimental procedures. Linear regression coefficients were typically ≧0.98. The maximum val-

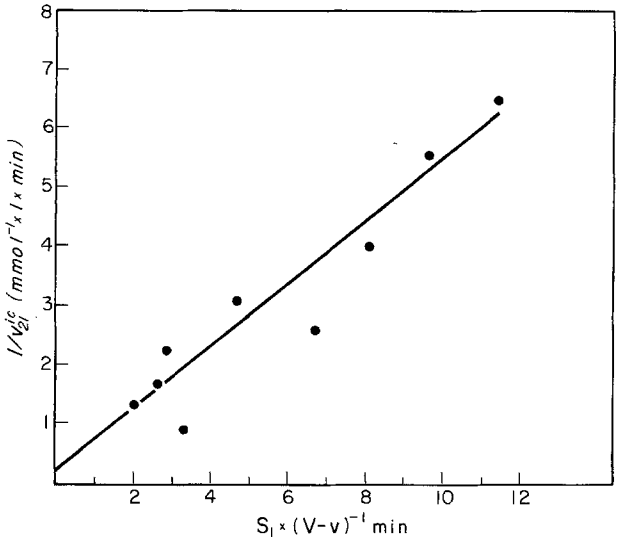

Fig. 12. Test for the presence of an unstirred layer on the outside of the red blood cell membrane according to Lieb and Stein (1974*b*). Data from infinite-cis efflux (ic_{21}) experiments [v_{21}^{ic} and estimated maximum velocity V_{21}^{ic} (*cf.* Table 4)] were plotted as $(v_{21}^{ic})^{-1}$ *vs.* $S_1^B \times (V_{21}^{ic} - v_{21}^{ic})^{-1}$, where S_1^B is L-leucine concentration in the bulk solution. *Ordinate:* mmol^{-1} × liter × min. *Abscissa:* minutes. The intercept on the abscissa is equal to P_1^{-1}, where P_1 is the permeability coefficient of the unstirred layer. The results of linear regression analysis are presented in Table 5

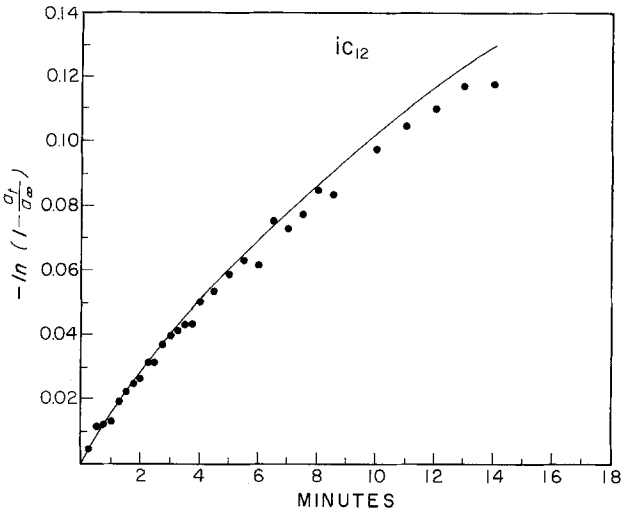

Fig. 13. L-leucine infinite-cis influx (ic_{12}) experiment in human red blood cells (25 °C, pH 7.4, hematocrit: 0.6 %). The initial intra- and extracellular concentration of L-leucine was 0 and 50 mM. *Ordinate:* $-\ln(1-a(t)/a_\infty)$, where $a(t)$ and a_∞ is the cellular concentration of ^{14}C-L-leucine at time zero and at equilibrium. *Abscissa:* time in minutes. Experimental data are indicated by (●). The time course curve predicted from the simple carrier model of Lieb and Stein (1974*b*) based on the results of Table 1 is shown for a comparison. *See text* for further explanation

Table 5. Testing and characterizing the simple carrier model of Lieb and Stein (1974*b*): testing for the presence of an unstirred layer on the outside of the red cell membrane[a]

Series of ic_{21} experiments	Slope of regression line (mM^{-1}) (±SD)	Intercept on the abscissa (min) (±SD)	Linear regression coefficient	Predicted value of slope (mM^{-1}) (±SD)
I	0.490±0.074	−1.00±1.13	0.95	0.272±0.047
II	0.338±0.041	−2.33±1.37	0.94	0.229±0.036
III	0.530±0.070	−0.13±0.95	0.95	0.296±0.070
Mean±SEM	0.453±0.058	−1.15±0.64		0.266±0.020

[a] The reciprocal of the flux v_{21}^{ic} measured in infinite-cis efflux experiments (*cf.* Table 4) was plotted against $S_1^B \times (V_{21}^{ic} - v_{21}^{ic})^{-1}$, where S_1^B is L-leucine concentration in the bulk solution. The slope of the regression line is predicted to be equal to $R_{ee} \times V_{21}^{ic} \times K_{00}^{-1}$ and the intercept on the abscissa to P_1^{-1} for the simple carrier model, where R_{ee} and K_{00} are parameters of the model, and P_1 the permeability coefficient of the unstirred layer. SD = standard deviation; SEM = standard error of the mean.

ue of the initial fractional rate coefficient from ic_{21} experiments was determined by linear regression of the reciprocal of the initial fractional rate coefficient ($=k^{-1}(min)$) *vs.* the concentration of L-leucine in the medium (Fig. 11). From the intercept on the ordinate an estimate of the fractional rate coefficient at 0 mM L-leucine in the medium was obtained, and used when calculating the initial rate of the maximum velocity V_{21}^{ic} from ic_{21} experiments. Results from three series of experiments are presented in Table 4. Mean values of the kinetic parameters (±SEM) were $V_{21}^{ic} = 0.925 \pm 0.069$ mmol × liter^{-1} × min^{-1} and $K^{ic} = 1.98 \pm 0.20$ mM.

The data from ic_{21} experiments were finally transformed to test for the presence of an unstirred layer at the outer side of the membrane as suggested by Lieb and Stein (1974*b*). From the simple carrier model a plot of $(v_{21}^{ic})^{-1}$ *vs.* $S_1^B (V_{21}^{ic} - v_{21}^{ic})^{-1}$ is predicted to be linear with an intercept on the abscissa equal to P^{-1}, where P is the permeability coefficient (min^{-1}) of the unstirred layer (see Fig. 12). S_1^B is L-leucine concentration in the bulk solution. Results from linear regression analysis are presented in Table 5. High values of the regression coefficients were obtained, and the estimated values of P^{-1} were not found to be significantly different from zero. Thus, there was no evidence for the presence of an unstirred layer on the outer side of the membrane.

Infinite-cis Influx (ic_{12}) Experiments

Eilam and Stein (1974) suggested following the time course of L-leucine influx from an "infinitely" high concentration of L-leucine in the medium when performing ic_{12} experiments, in order to compare this curve with a curve predicted from results of zero-

trans and equilibrium-exchange experiments. The latter curve is obtained by integration of the net flux equation for transport (*see* Appendix) as shown by Eilam and Stein (1974). The first part of the time course curve from an ic_{12} experiment is shown in Fig. 13.

Discussion

Evidence for Michaelis-Menten Kinetics

When results from zt_{12}, zt_{21} and ee_{21} experiments were examined for the goodness of fit by different kinetic models as advocated by Atkins and Gardner (1977), no statistical evidence for the presence of a nonsaturable route in addition to the carrier pathway was found. This is in agreement with the study of Hoare (1972*a*). As a permeability of finite magnitude for L-leucine has been measured in liposomes (Klein, Moore & Smith, 1971) and as low affinity transport of L-leucine via other amino acid transport systems than the L-system may occur (Rosenberg et al., 1980; Young et al., 1980) a three-parameter model might have been expected as the proper kinetic model. However, Hoare (1972*a*) has argued that a linear component of small magnitude compared to the carrier component may only be demonstrated by using very high concentration of substrate thus changing either osmotic pressure or ionic strength. Hoare took advantage of a pronounced decline of K_{12}^{zt} at low temperature and at 2.5 °C, was able to measure influx at L-leucine concentrations exceeding K_{12}^{zt} by a factor of 60. A statistically significant linear component contributing about 1% to the total flux at low concentrations of substrate was found by nonlinear regression analysis. If both components exhibit the same temperature dependence, the relative magnitudes of the components corresponded fairly well to the three-parameter regression solution of the present study. However, if a nonsaturable route is assumed to be present, it was not found possible in the present study to reliably estimate the contribution to the membrane permeability for L-leucine by this transport pathway.

At lower concentrations of L-leucine than the range applied for estimation of the kinetic parameters (0.3–50 mM), identical values of the unidirectional fluxes were measured in the above-mentioned experimental procedures. Furthermore, the fluxes were not different from predicted values, and no second-order kinetics was indicated at low concentrations of L-leucine (*cf.* Fig. 6). Hence, following the principle of Occam a two-parameter model was considered appropriate to account for the kinetic data in the concentration range studied (0.002–68 mM).

Testing the Simple Pore and Carrier Models of Lieb and Stein

A "simple pore" model consists of a continuous passage through the membrane, open at both ends and (when occupied) accessible simultaneously to substrate at both sides of the membrane, whereas the simple carrier is defined by the property of being alternately in contact with the solutions bathing the two sides of the membrane. For both models, only one particle is bound at a time (*see* Appendix). A Michaelis-Menten relation between the initial rate of the unidirectional flux and substrate is predicted by both models in the absence of an unstirred layer, i.e. a layer of finite permeability to the substrate giving a different concentration in the immediate vicinity of the binding site in the membrane as that in the bulk solution, when uncharged compounds are considered.

Identical fluxes at low concentrations ($S \ll K$) in zt_{12}, zt_{21} and ee_{21} experiments are predicted by both models as $V_{12}^{zt}/K_{12}^{zt} = V_{21}^{zt}/K_{21}^{zt} = V_{21}^{ee}/K_{21}^{ee} = 1/K_{00}$ (pore) or $1/K_{00}R_{00}$ (carrier). The value of R_{00}, i.e. the sum of resistances experienced by the free pore/carrier as it crosses the membrane in a cyclic fashion in the absence of substrate, provides a test to distinguish between the simple pore and carrier model as a value of $R_{00}=0$ is predicted by the former model and a value of $R_{00}>0$ by the latter. An estimate of R_{00} from the experiments gives R_{00}: 1.763 ± 0.074 (SD) $\text{mmol}^{-1} \times \text{liter} \times \text{min}$ thus indicating a rejection of the simple pore model, a model not considered by Hoare (1972*a*, *b*). However in agreement with Hoare no evidence against the simple carrier model was found from the results of the experimental procedures considered. To decide whether more complex models than the simple carrier are needed, various infinite experiments were performed.

In infinite-trans influx experiments considering a carrier model the net flux of labeled substrate is predicted to vanish at a concentration ratio of tracer $S_1^+/S_2^+ = (K_{00}+S_1)/(K_{00}+S_2)$ (Eq. 37 in Lieb & Stein, 1974*b*). Hence, considering the it_{12} experiment shown in Fig. 7, for a mean value of $K_{00}=2.16$ (mM), $S_2=17.6$ (mM) is obtained from Eq. 37. This value of S_2 can be compared with the cellular concentration of L-leucine after 40 min of transport, when calculated from the net flux equation (*cf.* Appendix). The calculated value of 21.9 mM is in reasonable agreement with the value $S_2=17.6$ mM giving a zero net flux of tracer. Furthermore, the accumulation of tracer apparently driven by the gradient of unlabeled substrate ("countertransport") provides a crucial criterion for rejection of the simple pore model.

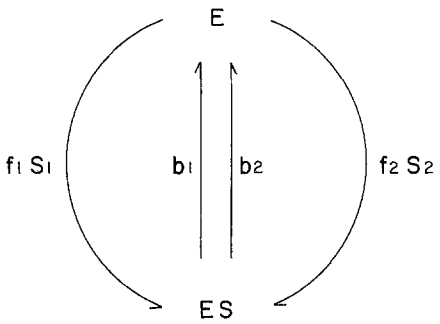

Fig. 14. Simple one-site pore model of Lieb and Stein (1974*a*)

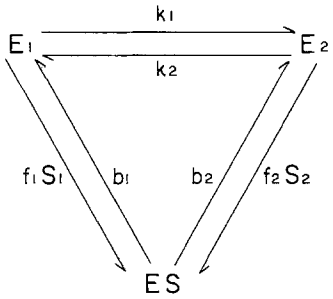

Fig. 15. Simple one-complex carrier model of Lieb and Stein (1974*b*)

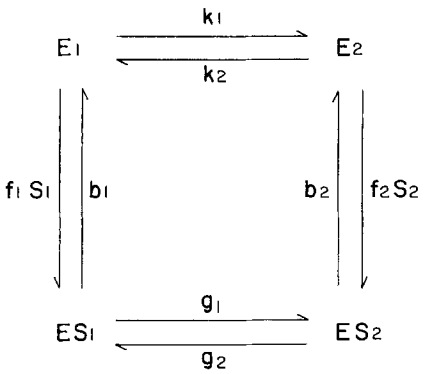

Fig. 16. Simple two-complex carrier model of Lieb and Stein (1974*b*)

Based on the requirement of consistency among kinetic parameters, the following predictions from the simple carrier model can be tested (*cf.* Appendix):

$$K_{12}^{it} = K_{21}^{ic} = K_{00} R_{21} R_{ee}^{-1} = 3.79 \pm 0.33 \text{ (SD) mM} \quad (1)$$

$$V_{21}^{ic} = V_{21}^{zt} = R_{21}^{-1} = 0.922 \pm 0.015 \text{ (SD) mmol} \times \text{liter}^{-1} \times \text{min}^{-1} \quad (2)$$

$$V_{12}^{it} = V_{21}^{ee} = R_{ee}^{-1} = 1.613 \pm 0.041 \text{ (SD) mmol} \times \text{liter}^{-1} \times \text{min}^{-1}. \quad (3)$$

K_{12}^{it}, K_{21}^{ic}, V_{21}^{ic} and V_{12}^{it} were all of the predicted order of magnitude. K_{12}^{it} and V_{21}^{ic} were within the predicted range, but when tested by a *t*-test K_{21}^{ic} and V_{12}^{it} were found smaller than K_{12}^{it} and V^{ee}, respectively ($p < 0.05$).

Infinite-trans (it_{21}) experiments have not been performed as the initial rate of the unidirectional flux cannot be reliably measured because of an immediate elevation of the concentration of L-leucine in the cells by influx at the maximum rate.

Infinite-cis influx (ic_{12}) experiments may provide further evidence for the validity of the carrier model. In Fig. 13 an uptake curve from an ic_{12} experiment and a curve predicted from integration of Eq. (3) of the Appendix as shown by Eilam and Stein (Eq. 72, 1974) are compared. The crucial comparison involves the first part of the curve, as the rate of influx rapidly declines with rising cellular concentration of L-leucine. Within the experimental error the curves were identical.

The asymmetry factor Q is defined as $Q = V_{21}^{zt}/V_{12}^{zt} = K_{21}^{zt}/K_{12}^{zt}$. From Table 1 a mean value of Q ($\pm$SD) $= 1.22 \pm 0.14$ is obtained. Hence at 25 °C a symmetrical transport mechanism is operating.

The following predictions have been derived by Cabantchik and Ginsburg (1977) and by Ginsburg (1978) from the simple carrier model:

$$Q + 1 \geqq 2(K_{21}^{zt} K_{21}^{ic-1})^{\frac{1}{2}} \quad (4)$$

$$Q + 1 \geqq 2(R_{00} R_{ee})^{\frac{1}{2}} / R_{21}. \quad (5)$$

Inserting the appropriate values $2.22 \pm 0.25 \geqq 2.64 \pm 0.16$ (Eq. 4) and $2.22 \pm 0.25 \geqq 1.92 \pm 0.06$ (Eq. 5) were obtained. Thus, no rejection of the carrier model was indicated.

The presence of an unstirred layer on either side of the membrane was not indicated from zt_{12} and zt_{21} experiments, as Michaelis-Menten equations were found in both kinds of experiments, even when low concentrations of L-leucine were applied (*cf.* Lieb & Stein, 1974*b*). Data from ic_{21} experiments can, however, be transformed to test directly for the presence of an unstirred layer at the outer side of the membrane:

$$(v_{21}^{ic})^{-1} = R_{ee} V_{21}^{ic} K_{00}^{-1} (S_1^B (V_{21}^{ic} - v_{21}^{ic})^{-1} - P_1^{-1})$$

where P_1 (min^{-1}) is the permeability coefficient of the unstirred layer and S_1^B L-leucine concentration in the bulk solution (Lieb & Stein, Eq. 19, 1974*b*). When $(v_{21}^{ic})^{-1}$ is plotted against $S_1^B (V_{21}^{ic} - v_{21}^{ic})^{-1}$ a straight line with $(P_1)^{-1}$ as the intercept on the abscissa is predicted. The slope of the line provides a further test of the carrier model. Regression coefficients $\geqq 0.94$ were obtained by linear least-square regression analysis, and there was no indication of the presence of an unstirred layer at the outer side of the membrane as "infinite" high values of P_1 were calculated (Table 5). However, the mean slope of the regression lines (0.453 ± 0.058, SEM) was greater than

the predicted value (0.266 ± 0.020, SEM) ($p < 0.05$).

A few deviations from the predictions of the simple carrier model have been found. All were of a moderate magnitude. They may be due to a variation introduced by performing the various experimental procedures at separate periods of time, or they may represent chance findings due to the testing of several predictions. However, it should be underlined that the statistical analysis, which has been applied in the present paper, has only been intended as a guide. A rigorous statistical analysis would require the availabiltiy of comprehensive statistical models derived explicitly to test the consistency of the carrier model according to the theory of Lieb and Stein (1974*a*, *b*). These models have not been derived.

In conclusion, a few deviations from the predictions of the simple carrier model have been found. They were not of the magnitude necessary to provide evidence for the rejection of the simple carrier model.

Interpretation of the Carrier Model in Terms of the Lieb and Stein Formalism

The kinetic model of Hoare is in several aspects similar to the simple carrier model of Lieb and Stein, and some of Hoare's main conclusions concerning the carrier mechanism are valid from the viewpoint of Lieb and Stein.

Based on the two-complex carrier model Hoare (1972*a*) concluded that at 25 °C (a) the carrier is about equally distributed between the membrane surfaces at equilibrium, (b) the carrier reorientation process is rate determining, and (c) the binding of L-leucine to the carrier increases the rate of carrier reorientation.

The first conclusion is also valid in terms of the Lieb and Stein analysis (Ginsburg & Cabantchik, 1977), and its validity is independent of the temperature considered. Concerning the second statement, Stein and Lieb (1973) have argued that by a steady-state analysis the carrier-complex reorientation and the breakdown of the complex will both be merged in the analytic description of transport, i.e. b_1 and g_2 of the two-complex carrier model cannot be separated out. As shown by Eilam and Stein (1974) the experimental finding $V^{ee} > V_{12}^{zt}$ implies that both b_1 and g_2 must be larger than k_2 (*see* Appendix). Hoare's third statement can thus be considered valid, but the relative magnitudes of b_1 and g_2 are not known. Hence it remains undecided whether or not the translocation step can be considered rate-determining as claimed by Hoare.

In *ee* experiments the concentration ratio E_2/E_1 of free carrier is equal to the ratio k_1/k_2 (Ginsburg & Chabantchik, 1977). Maximum and minimum values for the ratio k_1/k_2 can be calculated from the experimentally determined kinetic parameter as shown by Lieb and Stein (Table III in 1974*b*). The following inequality is then obtained: $0.6 < k_1/k_2 < 2.8$. Lower limits can be obtained for the ratios of other rate constants giving $b_2/k_2 > 1.1$ and $b_1/k_1 > 0.75$

R_{00} is an essential parameter of the simple carrier model since for all substrates that share the same carrier identical values of R_{00} should be estimated. Uridine transport in human red blood cells has been kinetically analyzed by the Lieb and Stein approach (Cabantchik & Ginsburg, 1977), and at 25 °C uridine transport was found to be a simple carrier-mediated type of transport like L-leucine transport, but the uridine carrier was found highly asymmetrical. Comparing the values of R_{00} for uridine ($R = 2.255 \pm 0.157$) and for L-leucine ($R_{00} = 1.763 \pm 0.074$) the properties of the two carriers are suggested to be different.

The interaction of carrier and substrate when the distribution of substrate is not a variable is described by the parameter K_{00} (Stein & Lieb, 1973). For the one-complex model $K_{00} = k_1/f_1 + k_2/f_2$ (*cf.* Appendix). For different substrates of the same carrier, differences in probability to form carrier-complex will be reflected in the K_{00} values, which are to be considered when relative affinities of substrates are evaluated. In zt_{12} experiments the apparent affinity constant K_{12}^{zt} ($= 2.78 \pm 0.58$ mM) and "true" affinity constant K_{00} ($= 2.16 \pm 0.17$ mM) are identical.

In conclusion, the results from the previous kinetic study of Hoare (1974*a*) of L-leucine transport at 25 °C in human red blood cells have been confirmed, and no evidence for the presence of a high-capacity nonsaturable transport route has been found in the concentration range studied (0.002–68 mM). No crucial evidence has been obtained that the description of L-leucine transport at 25 °C requires a more complex kinetic model than a simple carrier model which displays symmetry properties. The presence of an unstirred layer need not be taken into consideration. At physiological concentrations of L-leucine, i.e. about 0.1 mM, the membrane permeability coefficient is about 1.9×10^{-7} cm/sec. In terms of current concepts of membrane structure the carrier kinetics could be executed by a protein with gating-type properties (Lieb & Stein, 1976). L-leucine transport at 25 °C thus represents a convenient system for investigating molecular mechanisms of carrier-mediated transport which displays symmetry properties.

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Appendix

The results of the present work have been analyzed according to the simple pore and carrier theory of Lieb and Stein (1974*a*, *b*). The main features of this analysis are summarized in this Appendix. The experimental parameters V^{zt}, V^{ee}, V^{it} and V^{ic} and the corresponding K parameters are expressed in terms of the parameters of the kinetic models, i.e. K_{00}, R_{00}, R_{12}, R_{21} and R_{ee}. These parameters are finally expressed in terms of the rate constants k, f and b of the three models considered. S_1 and S_2 are substrate concentrations at the two faces of the membrane, u the unidirectional flux per unit transport molecule and j_{12} the net flux across the membrane. Figure 14 represents a one-site pore; Fig. 15 represents a one-complex carrier; and Fig. 16 represents a two-complex carrier.

1. *Fundamental Flux Equations*

One-site pore:

$$u_{12}=\frac{S_1}{K_{00}+R_{12}S_1+R_{21}S_2} \tag{A.1}$$

Carrier:

$$u_{12}=\frac{K_{00}S_1+S_1S_2}{K_{00}^2R_{00}+K_{00}R_{12}S_1+K_{00}R_{21}S_2+R_{ee}S_1S_2} \tag{A.2}$$

$$j_{12}=u_{12}-u_{21}. \tag{A.3}$$

2. *Interpretation of Experimental Data in Terms of Basic Measurable Parameters*

Experimental procedure

Net fluxes

A. zero-trans efflux

$$V_{21}^{zt}=R_{21}^{-1} \qquad K_{21}^{zt}=K_{00}R_{00}R_{21}^{-1}$$

B. zero-trans influx

$$V_{12}^{zt}=R_{12}^{-1} \qquad K_{12}^{zt}=K_{00}R_{00}R_{12}^{-1}$$

C. infinite-cis efflux

$$V_{21}^{ic}=R_{21}^{-1} \qquad K_{21}^{ic}=K_{00}R_{21}R_{ee}^{-1}$$

D. infinite-cis influx

$$V_{12}^{ic}=R_{12}^{-1} \qquad K_{12}^{ic}=K_{00}R_{12}R_{ee}^{-1}$$

E. infinite-trans influx

$$V_{12}^{it}=R_{ee}^{-1} \qquad K_{12}^{it}=K_{00}R_{21}R_{ee}^{-1}.$$

Unidirectional fluxes

A. equilibrium-exchange

$$V_{12}^{ee}=V_{21}^{ee}=R_{ee}^{-1} \qquad K_{12}^{ee}=K_{21}^{ee}=K_{00}R_{00}R_{ee}^{-1}$$

$$R_{00}=R_{12}+R_{21}-R_{ee}.$$

Definition of basic molecular parameters in terms of rate constants

Kinetic model	Parameters of models			
	K_{00}	R_{12}	R_{00}	R_{ee}
One-site pore	$1/f_1+1/f_2$	$1/b_2$	0	$1/b_1+1/b_2$
One-complex carrier	$k_1/f_1+k_2/f_2$	$1/b_2+1/k_2$	$1/k_1+1/k_2$	$1/b_1+1/b_2$
Two-complex carrier	$k_1/f_1+k_2(b_2+g_2)/f_2g_2$	$1/b_2+1/k_2+(b_2+g_2)/g_1b_2$	$1/k_1+1/k_2$	$1/b_1+1/b_2+(b_2+g_2)/g_1b_2+(b_1+g_1)/g_2b_1$

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