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A KINETIC ANALYSIS OF L-TRYPTOPHAN TRANSPORT IN HUMAN RED BLOOD CELLS

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1. The initial rate of L-tryptophan transport as a function of the cellular substrate concentration in human red blood cells was studied in zero-*trans* (= net) efflux and equilibrium-exchange efflux experiments at 25°C, pH 7.4. 2 Efflux curves were resolved into two Michaelis-Menten components. Further evidence was found that L-tryptophan transport at physiological concentrations of substrate is mediated predominantly by a recently identified transport system, designated the T-system. 3. The results from a kinetic analysis according to the theory of Lieb and Stein ((1974) Biochim. Biophys. Acta 373, 178–196) were consistent with the T-system being a carrier-mediated type of transport.

Introduction

Five amino acid transport systems have been described in the human red blood cell membrane. They are an L-system for L-leucine, L-phenylalanine, L-methionine and L-valine [1–3], an Ly-system for L-lysine and L-arginine [3,4], a T-system for L-tryptophan [5] an ASC-system for L-alanine and L-cysteine [3] and a glycine-transporting system [6].

The kinetics of L-leucine transport has been characterized in detail [7–9]. When analysed according to the simple pore and carrier theory of Lieb and Stein [10,11], data from net flux, equilibrium-exchange and infinite experiments were consistent with carrier-mediated transport [9].

The presence of a major transport route for L-tryptophan (the T-system) distinct from the pathway of other large neutral amino acids (the L-system) was indicated mainly from the results of uptake experiments. As the resolution of transport components with overlapping substrate specificities is a difficult task, it was considered essential to provide further evidence for the existence in the red cell membrane of a T-system, distinct from the L-system, based on the kinetic theory of Lieb and Stein [10,11]. An important aspect of this theory is that a kinetic criterion is given for the existence of separate transport systems

In the present paper, results from efflux experiments at 25°C, pH 7.4, and previously published uptake experiments [5] are combined and analysed following the approach of Lieb and Stein.

Materials and Methods

DTNB, FDNB, Hepes, L-leucine, PCMBS, Tes and L-tryptophan were obtained from Sigma Chemical Co, St. Louis, MO; Instagel® from Packard Instrument Company, Inc., IL, U.S.A.; *N*-ethylmaleimide from Fluka AG. Buchs SG, Switzerland; phloretin from K and K Biochemicals, Plainview, NY, and SITS from BDH Biochemicals Ltd, Poole, U.K. Other chemicals were of analytical grade.

Abbreviations DTNB, 5,5′-dithiobis(2-nitrobenzoic acid), FDNB, 1-fluoro-2,4-dinitrobenzene; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid, PCMBS, *p*-chloromercuriphenylsulphonate, SITS, 4-acetamido-4′-isothiocyano-2,2′-stilbene disulphonate, Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid

L-[*methylene*-^{14}C]Tryptophan and L-[U-^{14}C]leucine were obtained from Radiochemical Centre, Amersham, U.K. Radiochemical purity was assessed by TLC in butanol/water/acetic acid.

The transport experiments, the calculation of the initial rate of the unidirectional efflux and the statistical analysis of the concentration-dependence curves are described in detail elsewhere [9]. In brief, heparinized blood from the same donor was washed four times in incubation medium (138 mM NaCl/5 mM KCl/1 mM $MgCl_2$/1 mM Na_2HPO_4/10 mM Hepes/7.5 mM Tes, pH 7.4, 25°C), discarding the buffy coat. In efflux experiments, the cells were loaded by incubation in medium containing labeled and unlabeled L-tryptophan and 10 mM D-glucose (3–3.5 h at 25 or at 37°C, haematocrit 30–40%). The intracellular concentration of labeled amino acid was measured. Cells packed by centrifugation were injected into 10–15 ml medium, while stirring vigorously. For zero-*trans* efflux experiments, the medium was isotonic, without L-tryptophan. For equilibrium-exchange experiments, the medium contained L-tryptophan at the same concentration as within the cells. At predetermined time intervals, the cells were separated from the medium by centrifugation in an Eppendorf Microcentrifuge 3220. Immediately afterwards, part of the supernatant was prepared for liquid scintillation counting (Packard Instrument Scintillation Spectrometer model 2425).

Amino acid uptake was measured in a similar way except that cells were separated from the incubation medium by centrifugation through dibutyl phthalate, followed by determination of the concentration of tracer within the cells.

Binding of labeled amino acids to cell constituents was measured by dialysis using washed, haemolysed red blood cells (original haematocrit 35–45%). 1.5 ml of the haemolysate was placed in small sacs of Visking tubing (Scientific Instrument Co.). The sacs were incubated for up to 5 h (at 37°C, stirring continuously) in 50 ml 0.1 mM L-tryptophan or L-leucine containing the labeled amino acids in order to determine the concentration of tracer in the sacs and in the media at steady state.

Calculations. L-[^{14}C]Tryptophan transport was analysed by applying a closed two-compartment model with a single value for the rate coefficient k (s^{-1}) of tracer exchange [12]. Thus,

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

where $a(t)$ is the activity (dpm/l) in the cells (influx experiments) or in the medium (efflux experiments) at the time t of sampling; a_0 (dpm/l) and a_∞ (dpm/l) the tracer concentration at time zero in the medium (caused by trapped extracellular medium between the cells) and at equilibrium. The rate coefficient was estimated in duplicate by linear least-square regression of $\ln(1 - a(t)/a_\infty)$ vs. t, each run consisting of three to five sample times. The standard error of the mean was typically less than 7%.

The permeability P (cm/s) for L-tryptophan is given by

$$k = P \cdot A(1/z_1 + 1/z_e)$$

where z_1 and z_e represent the volume (cm^3) of the intra- and extracellular compartments, and A is the area (cm^2) of the membrane ($1.42 \cdot 10^{-6}$ cm^2 [13]). In order to correct for L-tryptophan binding to cell constituents, a z_1 value of $1.63 \cdot 0.63$ (= % water/wet weight of cells (w/w) [9]) was applied (see Results).

The unidirectional flux u_{ab} (mmol/($cm^2 \cdot s^{-1}$)) 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-tryptophan in compartment a. In zero-*trans* experiments, only the initial part of the time course curve was applied.

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 [14] was performed by an IBM 370/165 computer. Data were fitted to a 2-, a 3- and a 4-parameter model:

$$u = V \cdot S\,(K + S)^{-1}$$
$$u = V \cdot S\,(K + S)^{-1} + K_d \cdot S$$
$$u = V_1 \cdot S\,(K_1 + S)^{-1} + V_2 \cdot S\,(K_2 + S)^{-1}$$

where u is the initial rate of the unidirectional flux (mmol $\cdot$ l^{-1} $\cdot$ min^{-1}), V the maximum velocity (mmol $\cdot$ l^{-1} $\cdot$ min^{-1}), 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-tryptophan (mM). Before fitting of the data, the fluxes (original unit: mmol · $(cm^2)^{-1}$ · s^{-1}) were converted to the unit of mmol/l cell water per min, as this unit has been used in previous studies on amino acid transport in red blood cells.

The variance of various ratios and mean values of kinetic parameters, which constitutes the testing and characterization of the simple carrier model of Lieb and Stein [11] were estimated as suggested by Cleland (see Ref. 15, Eqn. 18).

The nomenclature of Lieb and Stein has been applied in the present paper

Results and Discussion

The steady state distribution ratio of L-tryptophan across the red cell membrane (mmol/l cell water : mmol/l medium) was 1.67 ± 0.02 (S.E.) (n = 31) at 25°C, and 1.60 ± 0.03 (S.E.) (n = 9) at 37°C, at final medium L-tryptophan concentrations of 0.02–18.6 mM The ratio did not correlate with substrate concentration, and was not affected by omitting D-glucose from the incubation medium or by increasing the incubation period to 48 h TLC indicated that there was no appreciable metabolism of L-tryptophan.

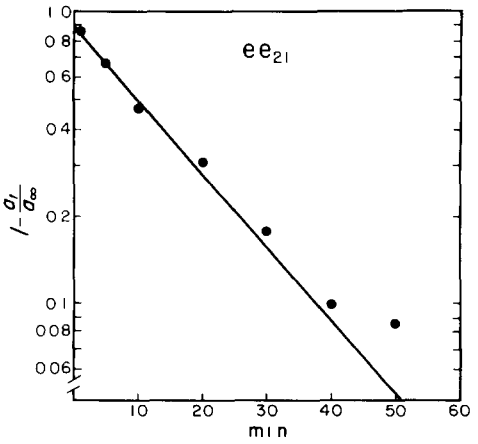

Fig. 1. Equilibrium-exchange efflux (ee_{21}) experiments in human red bloos cells at 0.04 mM L-tryptophan (25°C, pH 7.4, haematocrit 1%). Ordinate (log axis): $(1 - a_t/a_\infty)$, where a_t and a_∞ are L-[^{14}C]tryptophan concentrations in the medium at time t and at equilibrium. Abscissa time in min. The experiments were performed as previously described [5,9]

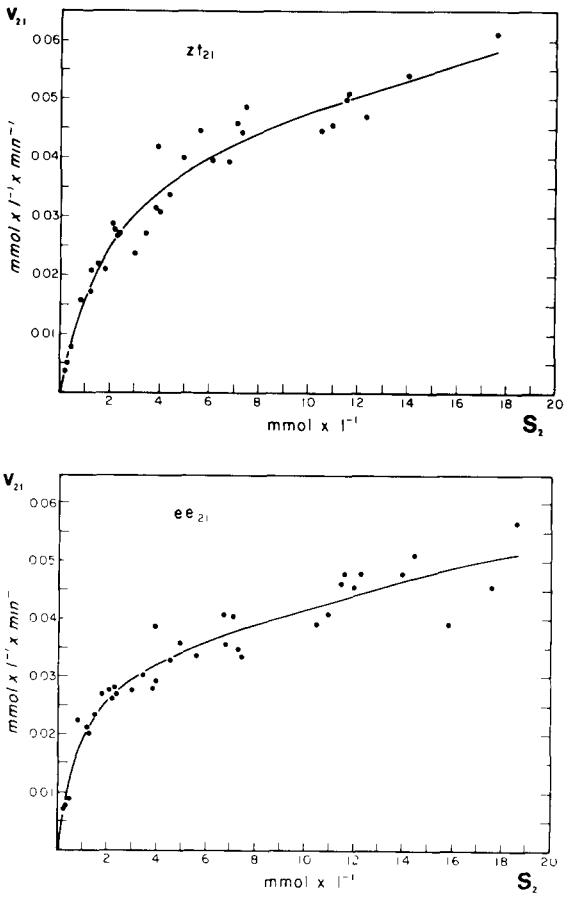

Fig 2 The initial rate of the unidirectional efflux (v_{21}) as a function of the cellular concentration of L-tryptophan from (a) zero-*trans* (zt_{21}) and (b) equilibrium-exchange (ee_{21}) efflux experiments in human red blood cells (25°C, pH 7 4). Ordinate mmol l^{-1} min^{-1}. Abscissa mM The experiments were performed as previously described [5,9].

The distribution ratio across the red cell membrane for L-tryptophan in human blood, after correction for albumin-binding, is reported to be lower [16] and higher [17] than unity. Recently, using blood from normals and schizophrenic patients, a ratio of 1.9 ± 0.6 (S.D.) was determined [18].

Binding of L-tryptophan to cell constituents sufficient to account for the observed distribution ratio across the red cell membrane was found in uptake experiments of L-tryptophan (0.1 mM) into dialysis

sacs containing haemolysed red cells (data not shown).

The time course of an equilibrium-exchange experiment at 0.04 mmol L-tryptophan per l cell water is shown in Fig. 1. Because the small deviation from a monoexponential fit can be attributed to heterogeneity of the rate constants of exchange (as in similar L-leucine efflux experiments [9]), only one cellular compartment was indicated.

Hence, the apparent higher cellular concentration of L-tryptophan (mmol/l cell water) at steady state was assumed to be due to binding to cell constituents, a finding which has been reported for several different compounds, i.e., serotonin, epinephrine and various drugs [19].

Concentration-dependence curves for L-tryptophan efflux

The concentration-dependence curves of the unidirectional flux of L-tryptophan from zero-*trans* (zt_{21}) and equilibrium-exchange (ee_{21}) efflux experiments are shown in Fig. 2a and b. Nonlinear regression analysis, fitting a 3-parameter model (cf. Ref. 5) to the efflux data are presented in Table I, and compared with previously published results from zero-*trans* (zt_{21}) experiments [5].

Kinetic parameters of the same order of magnitude were found in all experimental procedures considered.

The apparent linear component

Zero-*trans* influx experiments indicated that the linear component of transport represents L-tryptophan uptake via the L-system carrier [5]. An apparent inhibitor constant K_I ($= K_{12}^{zt}$) of 40 mM with respect to the L-system was estimated from *cis*-inhibition experiments of L-leucine uptake.

An inhibition of L-leucine uptake at 0.025 mM was demonstrated when L-tryptophan was present at the *trans* side of the membrane (Fig. 3). The order of interaction (m) and the L-tryptophan concentration giving a 50% inhibition ($K_{50\%}$) was estimated by linear regression analysis according to the equation

$$\log((v - v_i)/v_i) = m \log I - m \log K_{50\%}$$

where v and v_i are L-leucine influx in the absence, or prescence, respectively, of L-tryptophan at a concentration I in the cells [20] Estimates of m and $K_{50\%}$ were 1.04 ± 0.12 (S.D.) and 5.81 ± 1.24 (S.D.) mM, respectively. Transport via the L-system thus inhibited first-order interaction with respect to the substrate [20] in agreement with the results from the kinetic analysis of L-leucine transport [9].

Applying the kinetic analysis of Devés and Krupka [21], which is an extension of the Lieb and Stein analysis of one substrate transport [11], a K_{21}^{zt} value of 5.0 nM for L-tryptophan transport via the L-sys-

TABLE I

Kinetic parameters were obtained from nonlinear least-square regression analysis [14] of initial efflux rate vs. intracellular L-tryptophan concentration from zero-*trans* (zt_{21}) and equilibrium-exchange experiments (ee_{21}) in red blood cells (25°C, pH 7.4). Values are ±S.D. (n) where n represents the number of individual substrate concentrations used. Data from 5–6 series of experiments were pooled. A 2-parameter model ($v = V \cdot S/(K + S)$) and a 3-parameter model ($v = V \times S/(K + S) + K_d \cdot S$) were considered, where V is the maximum celocity, K the half-maximum constant, K_d the rate coefficient of a nonsaturable component, units as given in the table. Before fitting the 2-parameter model, the fraction of the flux mediated by the L-system ($V = 0.01$ mmol $\cdot$ l^{-1} $\cdot$ min^{-1}, $K = 5$ mM) was subtracted from the data, as described in the text. Kinetic parameters from zt_{21} experiments previously published [5] have been included in the table for comparison.

Experimental procedure	Number of parameters	V (mmol $\cdot$ l^{-1} $\cdot$ min^{-1})	K (mM)	K_d (min^{-1})	Concentration range (n) (mM)
zt_{21}	3	0.045 ± 0.007	1.88 ± 0.56	0.0010 ± 0.0004	0.2–17.6 (32)
	2	0.054 ± 0.003	3.05 ± 0.43	–	
ee_{21}	3	0.034 ± 0.003	0.82 ± 0.24	0.0010 ± 0.0002	0.2–19.6 (36)
	2	0.042 ± 0.002	1.66 ± 0.27	–	
zt_{12}	3	0.059 ± 0.011	3.53 ± 1.31	0.0019 ± 0.0003	0.3–40.0 (20)

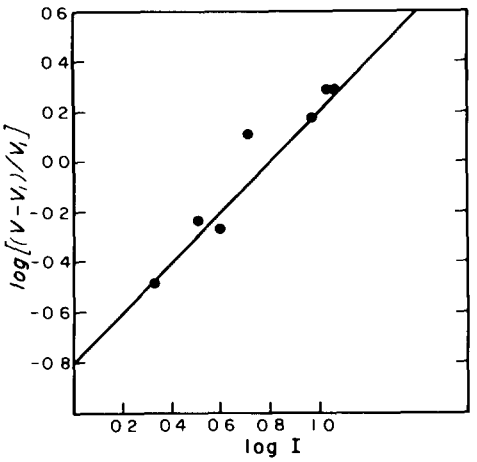

Fig 3. Inhibition of L-leucine uptake at uptake at 0 025 mM (25°C, pH 7.4) into human red blood cells by L-tryptophan at a cellular concentration of I (mM). Ordinate: $\log((v - v_i)/v_i)$, where v and v_i are the uptake rates of L-leucine in the abscence and in the presence of inhibitor in the cells. Abscissa $\log I$. The experiments were performed as previously described [5,9]

tem of was obtained from the *trans*-inhibition experiments. $V_{21}^{zt} = 0.0095$ mmol · l^{-1} · min^{-1} was then calculated from K_d of the linear uptake component (cf. Table I), as for carrier-mediated transport $V_{12}^{zt}/K_{12}^{zt} = V_{21}^{zt}/K_{21}^{zt}$ ($= K_d$) [11]. Thus, in contrast to the influx experiment, a kinetic model involving two Michaelis-Menten components in parallel was required to account for the net efflux data over the cellular L-tryptophan concentration range 0.02–17.6 mM.

Reliable estimates of kinetic parameters from efflux experiments when fitting a 4-parameter model, could not be obtained because of the scatter of data at high substrate concentrations.

L-Tryptophan transport assumed mediated via the L-system displayed asymmetrical properties with an asymmetry factor $Q = V_{21}^{zt}/V_{12}^{zt} = 0.13$. In contrast, $Q = 1.22$ has been reported for L-leucine transport via the L-system [9].

It should be underlined that at physiological concentrations (less than 0.1 mM) less than about 10% of L-tryptophan transport is mediated by the L-system.

The saturable component

The fraction of the efflux rate which could be accounted for by transport of L-tryptophan via the L-system was substracted from the data, assuming $V_{21}^{zt} = V_{21}^{ee} = 0.01$ mmol · l^{-1} · min^{-1} and $K_{21}^{zt} = K_{21}^{ee} = 5$ mM (cf. Fig. 2 and Table I). A 2-parameter model was then fitted by nonlinear regression analysis. As shown in Table I, kinetic parameters for the saturable component were virtually identical when comparing the 2- and 3-parameter regression solutions.

The maximum rates of L-tryptophan efflux were of an order of magnitude smaller than corresponding rates of L-leucine efflux via the L-system, while the half-maximum concentrations constants were of the same magnitude [9].

In contrast to similar L-leucine experiments, giving values of V_{21}^{ee} and V_{21}^{zt} of 1.61 and 0.92 mmol · l^{-1} · min^{-1}, respectively [9], no increase of the rate of L-tryptophan efflux by the presence of substrate in the medium was demonstrated (cf Table I).

To decide whether the Michaelis-Menten component, designated the T-system [5], might be a carrier-mediated type of transport, some predictions from the simple carrier model of Lieb and Stein [10,11] were tested.

The prediction of $R_{00} = 1/k_1 + 1/k_2 > 0$, where k_1 and k_2 are rate constants of the translocation of the empty carrier (cf. Fig. 4), was fulfilled by the results of the kinetic experiments, as an estimate of $R_{00} = 11.66 \pm 3.43$ mmol^{-1} · l · min was obtained.

At low concentrations of substrate ($S << K^{zt}$), identical values of experimentally determined unidirectional fluxes u_{ab} (mmol · l^{-1} · min^{-1}) should be measured in the three different experimental procedures considered [11]. Furthermore u_{ab} should be related to the substrate concentration S by

$$u_{ab} = V/K \cdot S,$$

where V/K are the ratio of the kinetic parameters.

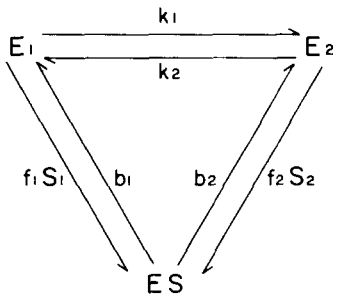

Fig. 4 The one-complex simple carrier model of Lieb and Stein [9,10], k_1, k_2, b_1, b_2 are molecular rate constants; S_1 and S_2 are concentrations of substrate in compartment 1 and 2.

TABLE II

Test of the simple carrier model. Comparison of ratios of experimental kinetic parameters (V/K (min^{-1})) from Table I and the fractional rate coefficient of transport k (min^{-1}) measured at low concentrations of L-tryptophan (0.04–0.1 mM) relative to the concentration range applied for estimation of kinetic parameters (haematocrit 1%). Values of k are mean ± S.E., the number of experiments given in parentheses. Equal V/K ratios are predicted from the simple carrier model comparing the regression solution from zt_{12} influx experiments and the 2-parameter regression solution of efflux experiments. The rate coefficient k is predicted to be equal to $V/K + K_L$, where K_L represents L-tryptophan transport via the L-system. See text for experimental details.

Experimental procedure	V/K (min^{-1})	$V/K + K_L$ (min^{-1})	k (min^{-1})
zt_{21}	0.0177 ± 0.0026	0.0197	0.0228 ± 0.0028 (7)
ee_{21}	0 0253 ± 0.0042	0.0273	0.0285 ± 0.0020 (8)
zt_{12}	0.0167 ± 0.0069	0.0186	0.0375 ± 0.0064 (8)

When these predictions were tested from the results of Table II, no decisive evidence for a rejection of the simple carrier model was obtained.

Various infinite experiments, i.e., applying very high concentrations of substrate ($S >> K^{zt}$) at one of the faces of the membrane are needed to provide further evidence for the validity of the carrier model [11]. However, as a substantial fraction of L-tryptophan transport at high concentrations is mediated via the L-system, it would probably be difficult to obtain reliable estimates of the kinetic parameters, thereby limiting the importance of these experiments.

As the present kinetic analysis of L-tryptophan transport via the T-system provides no evidence for rejection of the simple carrier model, and as the estimated value of R_{00} (11.66 $mmol^{-1} \cdot l \cdot min$) for L-tryptophan transport via this transport route differs by an order of magnitude from that of L-leucine (1.76 $mmol^{-1} \cdot l \cdot min$) via the L-system, it is confirmed that the T- and L-systems are different carrier-mediated systems.

Inhibition of L-tryptophan transport

L-Tryptophan efflux at 0.1 mM was strongly inhibited (95%) by 0.25 mM phloretin, a pronounced inhibitor of several carrier-mediated transport systems probably due to an interaction with membrane proteins [22].

L-Tryptophan uptake (0.1 mM) was measured after incubation of red cells at 37°C for 20 min with different covalent modifiers of transport pathways [23], followed by washings of the cells before the uptake experiments (37°C, triplicate runs with S.E. typically less than 5–10%). Strong inhibition of transport (80–95%) was obtained by an amino-reactive reagent (2 mM FDNB) and by SH-reagents (5 mM *N*-ethylmaleimide, 1.0 mM PCMBS). Another SH-reagent DTNB (2.5 mM) gave a moderate inhibition (25%), while SITS (0.6 mM), a specific anion-transport inhibitor, did not inhibite L-tryptophan transport. The different inhibition capacity for FDNB and DTNB may be due to separate sites of action at membrane proteins.

Thus, both amino-groups and SH-groups appear to be involved at the site of action of the T-system pathway.

Conclusion

Further evidence has been provided that at physiological concentrations of substrate, L-tryptophan transport across the membrne of the red blood cell is predominantly mediated by a transport system, designated the T-system, which is distinct from the carrier-mediated system of L-leucine transport. Results from net flux and equilibrium-exchange experiments were consistent with the T-system also being a carrier-mediated pathway. The physiological significance of the T-system remains to be elucidated.

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