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THE TRANSPORT OF L-LEUCINE IN HUMAN ERYTHROCYTES: A NEW KINETIC ANALYSIS

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SUMMARY

1. Data on the transport of L-leucine into human erythrocytes at 25° C shows (*a*) that the carrier re-orientation process is rate determining, (*b*) that the binding of leucine to the carrier increases the rate of carrier re-orientation and (*c*) that the carrier is, at equilibrium, about equally distributed between the membrane surfaces at 25° C.

2. These conclusions are reached through a new kinetic analysis of a simple carrier system, which involves no prior assumptions about the relative magnitudes of the rate constants, yet leads to usable kinetic equations. These equations allow the determination of the rate determining step, the calculation of the effect of bound substrate on the rate of carrier re-orientation and, in some cases, an estimate to be made of the equilibrium distribution of the carrier between the inner and outer membrane surfaces.

INTRODUCTION

The transport of glucose, and, to a small extent, amino acids into the human erythrocyte has been used in establishing the basis of the present theory of carrier-mediated transport (reviewed, for example, by Stein, 1967). However, although complete kinetic schemes have been derived for a range of possible carrier mechanisms (Jacquez, 1964), these have always been simplified by a number of critical assumptions before being applied to experimental data. These assumptions have usually been concerned with the nature of the rate-determining step, and with the symmetry of the carrier with regard to the inner and outer surfaces of the membrane. Consequently, no definite information is available on these important features of the mechanism, and the conclusions which have been drawn are in some degree dependent on the validity of the original assumptions. In this paper, a new form of the kinetic equations for the general case is

presented which is particularly easy to interpret, and the limitations imposed by various common assumptions are explored. The kinetic equations are applied to the non-concentrative transport of leucine in human erythrocytes to obtain as full a picture as possible of this carrier system.

The question of the structural and functional symmetry of the carrier for sodium-linked glycine transport in pigeon erythrocytes has been considered in some detail by Vidaver & Shepherd (1968). They have extended the useful practice, initiated by Levine & Stein (1966), of designating important ratios of rate constants by dimensionless constants, which can then be substituted into the kinetic equations of the system; this allows the effects of changes of these ratios to be distinguished from that of changes in the absolute magnitudes of the rate constants of the system. However, although Vidaver & Shepherd were able to demonstrate that their carrier system is definitely asymmetric, they could not assign unambiguous values to any of the ratios. In this paper, the symmetry of the leucine carrier in human erythrocytes is investigated, and in this rather simpler system some definite information can be obtained.

Previous studies by Winter & Christensen (1964) have established that there are two pathways for amino-acid transport into human erythrocytes, one specific for alanine and glycine, and the other specific for leucine, valine, and related amino acids. There appeared also to be a non-saturable pathway, giving an over-all kinetic relationship,

$$\text{Flux} = V.[S]/(K_m+[S])+D[S].$$

It was shown that in an erythrocyte containing initially equal intracellular and extracellular concentrations of leucine, an extracellular concentration of methionine could induce a transitory outward 'uphill' flux of leucine; in addition, comparison of the inward and outward fluxes indicated that the carrier system had an approximate functional symmetry with respect to the inner and outer faces of the membrane, although, as will be shown below, this does not necessarily correspond to a complete symmetry of all aspects of the system.

METHODS

Materials and reagents

Chemicals

Naphthalene, 2,5-diphenylisoxazole (PPO) and 1,4-di-2-(5-phenyloxazoyl)-benzene (POPOP) from Koch Light Laboratories Ltd. Dioxan from Fison Scientific Apparatus Ltd. All radiochemicals from the Radiochemical Centre, Amersham. Leucine (A grade) from Calbiochem. Ltd.

Balanced salt solution

1 volume of a solution containing 1·3 M sodium chloride, 0·05 M potassium chloride, and 0·01 M magnesium chloride and 1 volume of 0·25 M sodium phosphate buffer at pH 7·4 were diluted to 10 volumes with distilled water to give balanced salt solution.

Bray solution

60 g naphthalene, 4 g PPO, 0·2 g POPOP, 20 ml. methanol and 20 ml. ethylene glycol were made up to 1 l. with dixan and stored in the dark.

Source and preparation of erythrocytes

30 ml. samples of fresh human blood were added to 1·0 ml. of EDTA solution (disodium salt dihydrate, 37 g/l.) and stored at 2° C; samples were used within 14 days.

Before an experiment, the erythrocytes were freed from plasma and buffy coat cells by centrifugation, and then by twice suspending in 10 volumes of balanced salt solution, centrifuging for 10 min at 2000 *g*, and removing the supernatant and upper layer of erythrocytes. They were then suspended in 10 volumes of balanced salt solution at 37° C for 1 hr, isolated by centrifugation, and washed once more with 10 volumes of balanced salt solution at room temperature.

Entry experiments

The rate of entry of amino acids into erythrocytes containing no free amino acids was measured by the following procedure based on Winter & Christensen (1964).

(*a*) *Procedure.* 1·0 ml. of a suspension of washed erythrocytes in balanced salt solution (haematocrit $\sim$ 30%) were added to 2·0 ml. [^{14}C]amino acid in balanced salt solution [^{14}C content $\simeq$ 0·25 μCi]; the temperature was maintained $\pm$0·1° C in a thermostat bath. The first sample of 1·0 ml. was removed as soon as possible (usually in less than 15 sec) and pipetted into 10 ml. of balanced salt solution at 0° C to arrest the transport process. A second sample of 1·0 ml. was taken after a suitable time and treated similarly. The samples were then centrifuged for 10 min at 0° C and about 2000 *g*; the supernants were removed, and the pellets of erythrocytes washed twice by suspension in 10 ml. of balanced salt solution at 0° C, followed by centrifugation. Each pellet was then haemolysed by addition of 2·0 ml. water. The protein was precipitated by addition of 1·0 ml. trichloroacetic acid solution (300 g/l.) and centrifugation. 1·0 ml. of the supernatant was added to 10 ml. Bray solution and the radioactivity measured by scintillation counting (Nuclear-Chicago Unilux II Scintillation Counter).

Control experiments confirmed that the entry process was effectively terminated by dilution in the balanced salt solution. The tenfold dilution of the extracellular solution decreases the concentration gradient of leucine across the membrane very considerably, and measurements of the flux of leucine across the erythrocyte membrane at 0° C showed that the half-time for reaching equilibrium would be greater than 2 hr. Control experiments also confirmed that there was negligible co-precipitation of amino acid with the protein during trichloroacetic acid precipitation.

After removal of the second sample, 0·5 ml. of the remaining suspension were transferred to a haematocrit tube (a capillary tube of about 1·2 mm internal diameter with a wider upper portion of capacity about 1 ml.) and centrifuged at 0° C and 2000 *g* for 30·0 min. The tubes had been individually calibrated with mercury, so that

the haematocrit could be calculated directly from the height of the column of packed erythrocytes (S.E. of estimate in control experiments = ±2% of measured value).

The molar activity of the [^{14}C]amino acid was estimated by adding 0·05 ml. of the supernatant from the haematocrit tube with 1·0 ml. trichloroacetic acid solution (100 g/l.) to 10 ml. Bray solution and counting as described above.

(*b*) *Quenching corrections.* When the scintillation counter is set up as normally recommended for the channel ratio method of quench correction, it can be shown that the trichloroacetic acid solution containing the amino acid after protein precipitation quenches the total counts to about 50%, but, more important, the observed counts were very dependent on small variations in the quantity or composition of the trichloroacetic acid solution used. The following procedure was therefore adopted which, at the price of slightly greater over-all quenching, made the total counts very insensitive to fluctuations in the amount or composition of the trichloroacetic solution added, and therefore reduced the random error considerably.

Three vials were made up with identical contents of [^{14}C]leucine, containing respectively 0·95 ml. (vial A), 1·0 ml. (vial B) and 1·05 ml. (vial C) of 10% trichloroacetic acid solution. The three vials were then counted in succession and the gain on the instrument adjusted until vial B gave the greatest number of counts, and vials A and C were equal and slightly less. With this setting, therefore, a graph of observed counts versus volume of trichloroacetic acid solution added would show a maximum at 1·0 ml., and the fluctuations in the observed counts with volume of trichloroacetic acid solution added would be a minimum at this point. A second channel was then used to measure the quenching relative to this maximum by the normal channel ratio procedure; however, the quench correction required was rarely statistically significant, and never exceeded 1%.

(*c*) *Calculation of results.* Calculations of quench corrections, rates of transport, etc., were performed on an Elliot 4100 computer. The above experiments yielded, for each rate measurement, values for the haematocrit, molar activity of the amino acid, and uptake of ^{14}C by the erythrocytes after two time intervals. Assuming that the packed cell volume measured in the haematocrit tubes includes 5% of entrapped extracellular fluid, and that the intracellular water occupies 65% of the volume of the erythrocyte (Winter & Christensen, 1964), the intracellular concentration of amino acid could be calculated for each of the two samples taken, and the average rate of uptake (in mole/kg cell water.sec) computed from the gradient of the line drawn through the two values. The timing of the second sample was arranged so that the maximum observed intracellular concentration of amino acid was usually in the range 10–20% of the initial extracellular concentration, so that the measured rate corresponded reasonably well with the 'initial rate' approximation. When necessary, the second point was taken earlier, so that as little as 2% of the entry process was observed in some cases. Since the observed haematocrit values were usually in the range 7–10%, the extracellular concentration of amino acid could be regarded as constant during the course of the experiment. The statistical errors from the scintillation counting were combined to yield a total statistical error for the transport rate, which was routinely computed for each measurement; this rarely exceeded 3%.

(*d*) *The validity of the initial rate approximation.* The procedure described above yields the *average* entry rate during the first 10–20% of the entry process. This can only be equated to the initial rate if it can be shown that the inward flux is approximately constant during this period. It is, of course, possible that the accumulation of leucine inside the cell can interact with the carrier system, altering the entry rate as the process proceeds, and invalidating the initial rate assumption. This is particularly likely to occur when the concentrations of leucine exceeds the dissociation constant of the carrier-substrate complex at the inner membrane surface.

Fig. 1 shows the course of an entry experiment in which the external leucine concentration (9·9 mM) exceeded the K_m value for the carrier system under the conditions of the reaction ($K_m \simeq 2$ mM at 20° C). The results indicate that the entry rate is approximately constant over the first 15 % of the reaction, and so in this case the average entry derived from the 'two-point' procedure described above can be equated with the initial rate. The small intercept at zero time probably corresponds to extracellular leucine carried over after the washing step.

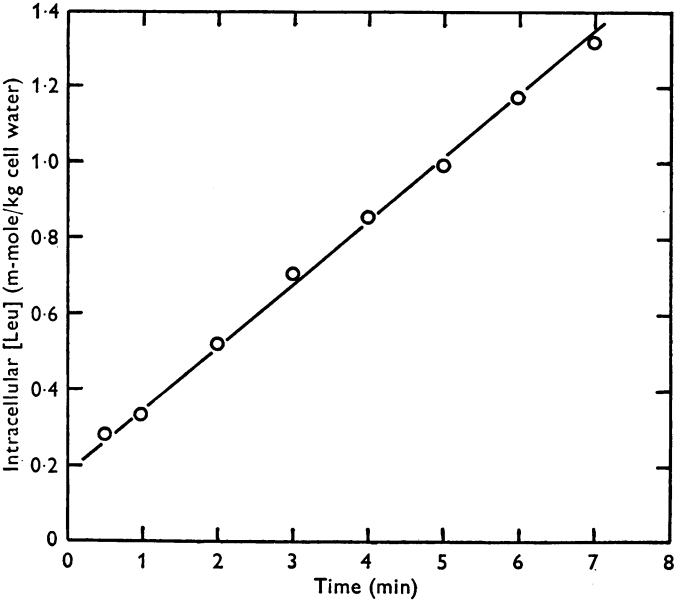

Fig. 1. The entry of L-leucine into human erythrocytes at 20° C. Extracellular [Leu] = 9·9 mM. Initial intracellular [Leu] = 0.

Fig. 2 shows a more testing case, in which the extracellular leucine concentration (9·0 mM) very considerably exceeds the value of K_m, which is about 0·4 mM under these conditions (i.e. 5° C). Towards the end of the experiment, the intracellular concentration of leucine is twofold greater than this K_m value. However, there is no indication that the entry rate has altered during the experiment. The most probable explanation is that the value of K_m is much smaller than the true dissociation constant of the carrier complex. In entry experiments, K_m is a complex function of the rate constants of the carrier system (see below) and could well be much smaller than the true dissociation constant.

Since the final intracellular leucine concentration does not exceed about twice the value of K_m in any entry experiment reported in this paper, these results show that the average entry rates measured by the 'two-point' procedure can be equated approximately with the initial entry rate. This type of experiment must necessarily produce a small systematic underestimate of the initial rate, but this is unlikely to exceed 15 %. Even in a system where the carrier is far from saturation, the average rate will be about 10 % less than the true initial rate when the transport process has proceeded 20 % towards equilibrium. Larger underestimates than this should produce

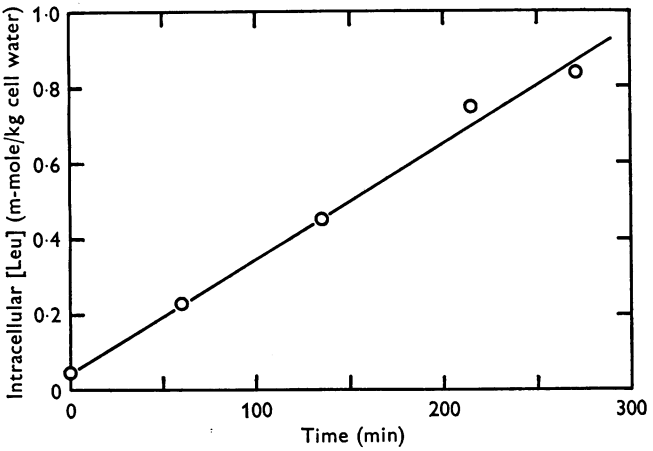

Fig. 2. The entry of L-leucine into human erythrocytes at 5° C. Extracellular [Leu] = 9·0 mM. Initial intracellular [Leu] = 0.

a perceptible curvature in uptake graphs such as Figs. 1 and 2, although the curvature corresponding to a 10 % underestimate is scarcely perceptible.

Exchange experiments

Exchange experiments differ from transport experiments in measuring the total flux of amino acid into erythrocytes when the internal and external concentrations are at equilibrium and equal (Winter & Christensen, 1964), rather than into erythrocytes containing zero concentration of amino acid. The procedures for the two types of experiment are very similar, and differ only as noted below.

Procedure. 1·0 ml. washed erythrocyte suspension (haematocrit 30 %) were added to 2·0 ml. unlabelled amino acid in balanced salt solution and incubated for 1 hr at 37° C (this time refers to leucine; if other amino acids are used, the time must be sufficient to allow the intracellular concentration to come to near equilibrium with the extracellular concentration, and may have to be altered if the amino acid transport rate differs substantially from that of leucine). The rate measurement was initiated by adding a small volume (25–50 μl.) of [^{14}C]amino acid solution (about 0·25 μCi at about 300 mCi/m-mole) to the extracellular fluid. After this point, the exchange and transport experiments were identical.

Exit experiments

In exit experiments, the erythrocytes are first preloaded with ^{14}C-labelled amino acid, then the extracellular amino acid is removed by washing and the outward flux into balanced salt solution is measured.

Procedure. 1·0 ml. washed erythrocyte suspension (haematocrit 30 %) was added to 2·0 ml. ^{14}C-labelled amino acid in balanced salt solution and incubated for 1 hr at 37° C. The solution was then cooled to 0° C, centrifuged, and 50 μl. of the supernatant removed and added with 1·0 ml. water to 10 ml. Bray solution to determine the activity of the amino acid. The cells were washed once with 10 ml. of balanced salt solution at 0° C, and then resuspended in 3·0 ml. of balanced salt solution at the

required temperature in a thermostat bath. Two 1·0 ml. samples were removed at appropriate times (as in transport experiments), added to 1·0 ml. of balanced salt solution at 0° C, and centrifuged rapidly at 0° C. 1·0 ml. of the supernatant was then removed for estimation of the ^{14}C content by counting in Bray solution. The outward fluxes were calculated using the same assumptions as for transport experiments.

Glossary of principal symbols used in kinetic analysis

C refers to carrier molecule;
D rate constant for non-saturable pathway (cf. eqn. (10));
k used to designate rate constants;
K_m Michaelis constant;
K (with other subscript) dissociation constant;
p ratio of re-orientation rate constants for full and empty carrier (cf. eqn. (1));
q ratio of inward and outward reorientation rates of empty carrier (cf. eqn. (2));
r^2 ratio of dissociation constants for substrate from carrier (cf. eqn. (3));
R the Gas constant;
s ratio of dissociation rates for substrate from carrier (cf. eqn. (5));
S refers to the substrate of the transport system;
T temperature;
V maximum flux (as in Michaelis–Menten equation);
W, X, Y ratios of V values defined in eqns. (12), (13) and (14).

Subscripts

b binding process;
c empty carrier;
d dissociation process;
i inside or inward;
o outside or outward;
s carrier-substrate complex.

RESULTS

Kinetic analysis

Fig. 3 shows the carrier system which has been used as the basis for analysing the data in this paper. It is of the type originally proposed by Widdas (1952) and which has subsequently been widely used. Previously, it has been necessary to simplify the model by making some arbitrary assumptions about, for example, the nature of the rate-determining step, before it can usefully be applied to experimental data. In this case, however, it is possible to start with no prior assumptions about rate-determining steps, or the relative magnitude of any of the rate constants.

Even the simple carrier model considered in this paper contains seven independent rate constants, and the kinetic experiments described below cannot provide independent values for each. Instead, they have been used to obtain various important ratios of rate constants which are defined below.

(i) *The effect of the binding of substrate on the carrier re-orientation rate.* To avoid arbitrarily using the inward or outward re-orientation rates in defining the ratio of the re-orientation rate constants of full and empty

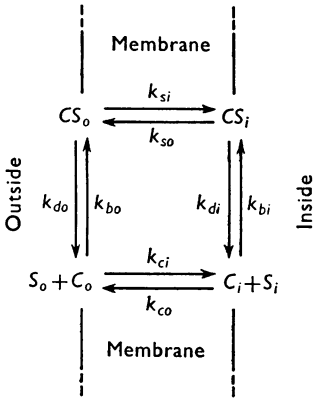

Fig. 3. A simple carrier mechanism.

carriers, two mean rate constants, k_s and k_c are defined as the harmonic means of the respective inward and outward rate constants.

$$2/k_s = 1/k_{si}+1/k_{so}, \quad 2/k_c = 1/k_{ci}+1/k_{co}.$$

Then

$$p = k_s/k_c, \tag{1}$$

p is therefore equivalent to the ratio r used by Levine & Stein (1966), except that they assumed that $k_{si} = k_{so}$ and $k_{ci} = k_{co}$.

(ii) *The distribution of the carrier at equilibrium.* Since there is no *a priori* reason for assuming that the carrier should be equally distributed between the inner and outer surfaces of the membrane, a distribution constant can be defined as follows

$$q = k_{ci}/k_{co}. \tag{2}$$

(iii) *The ratio of dissociation constants.* The dissociation constant of the carrier-substrate complex at the inner and outer surfaces of the membrane may differ, and this can also be expressed as a ratio

$$r^2 = \frac{k_{di}}{k_{bi}} \cdot \frac{k_{bo}}{k_{do}} = \frac{K_{si}}{K_{so}}. \tag{3}$$

(iv) *The distribution of the substrate-carrier complex at equilibrium.* Since the product of all the equilibrium constants, taken sequentially, must be unity in a closed loop such as the carrier system in Fig. 3, the equilibrium distribution of the substrate-carrier complex between the inner and outer surfaces of the membrane can be derived directly from the ratios already defined, giving:

$$q/r^2 = k_{si}/k_{so}. \tag{4}$$

(v) *The ratio of the dissociation rates.* The dissociation rate of substrate from the carrier-substrate complex may also differ between the two surfaces of the membrane, and this can be expressed by

$$s = k_{di}/k_{do}. \tag{5}$$

Three types of experiment have been used in this study. In *entry* experiments, the inward flux of substrate is measured when the initial intracellular concentration is zero. In *exit* experiments, the erythrocytes are preloaded with substrate, and the outward flux is measured when the initial extracellular substrate concentration is zero. In *exchange* experiments, the intracellular and extracellular concentrations of substrate are equal, and the inward flux is measured by adding a ^{14}C-tracer to the extracellular solution. The kinetic equations giving the flux of substrate in entry and exit experiments were derived by a straightforward steady-state procedure, based on the model in Fig. 3. In the case of exchange experiments, it was assumed that the distribution of the carrier between the membrane surfaces, and between free carrier and substrate-carrier complex, was at equilibrium and a steady-state procedure was then applied to the flux of the ^{14}C-tracer. In all three cases, the resulting kinetic equations showed that the flux was related to substrate concentration by a simple Michaelis–Menten equation, Flux $= [S].V/([S]+K_m)$, in which V and K_m were given by the following expressions.

For entry experiments. (The expressions for exit experiments can of course be obtained by substituting inward rate constant for the corresponding outward ones, and vice versa; $\{C\}$ = total carrier concentration in the membrane.)

$$V/\{C\} = \frac{k_{di}.k_{si}.k_{co}}{F}, \tag{6}$$

$$K_m = \frac{(k_{ci}+k_{co})}{k_{bo}}.\frac{G}{F}, \tag{7}$$

where

$$F = (k_{co}.k_{si}+k_{co}.k_{di}+k_{co}.k_{so}+k_{di}.k_{si}),$$

$$G = (k_{do}.k_{di}+k_{do}.k_{so}+k_{si}.k_{di}).$$

For exchange experiments.

$$V/\{C\} = \frac{k_{di}^2.k_{si}.k_{co}.k_{bo}.k_{do}}{G.H}, \tag{8}$$

$$K_m = \frac{k_{di}.k_{do}(k_{ci}+k_{co})}{H}, \tag{9}$$

where G = as above

$$H = (k_{bo}.k_{co}.k_{di}+k_{do}.k_{ci}.k_{bi}).$$

These expressions were then considerably simplified by considering the two cases in which either the substrate-carrier association–dissociation processes, or the carrier re-orientation processes, are rate-determining. After substituting in the ratios p, q, r^2 and s, defined above, the expressions shown in Table 1 were obtained (with $K_{so} = k_{do}/k_{bo}$). The applications of these expressions are discussed below.

TABLE 1. Expressions for V and K_m in terms of the rate constants in Fig. 3 $K_{so} = k_{do}/k_{bo}$; p, q and r^2 are defined by eqns. (1), (2) and (3). $\{C\}$ is the total concentration of carriers in the membrane

Type of experiment		Reorientation rate-determining	Association–dissociation rate-determining
Entry	$V/\{C\}$	$\frac{k_s}{2}\cdot\frac{(1+q/r^2)(1+q)}{pq(1+q/r^2)+(1+q)}$	$\frac{k_{di}}{1+q/r^2}\cdot\frac{q}{r^2}$
	K_m	$K_{so}\cdot\frac{(1+q)^2}{pq(1+q/r^2)+(1+q)}$	$K_{so}\cdot\frac{1+q}{1+q/r^2}\cdot(1+qs/r^2)$
Exchange	$V/\{C\}$	$\frac{k_s}{2}$	$\frac{k_{di}}{1+q/r^2}\cdot\frac{q}{r^2}\cdot\frac{1}{1+qs/r^2}$
	K_m	$K_{so}\cdot\frac{1+q}{1+q/r^2}$	$K_{so}\cdot\frac{1+q}{1+q/r^2}$
Exit	$V/\{C\}$	$\frac{k_s}{2}\cdot\frac{(1+q/r^2)(1+q)}{p(1+q/r^2)+(1+q).q/r^2}$	$\frac{k_{di}}{1+q/r^2}\cdot\frac{1}{s}$
	K_m	$K_{so}\cdot\frac{(1+q)^2}{p(1+q/r^2)+(1+q).q/r^2}$	$K_{so}\cdot\frac{1+q}{1+q/r^2}\cdot\frac{1+qs/r^2}{qs/r^2}$

The non-saturable pathway

The kinetic model discussed above predicts that for transport, exit and exchange experiments, the flux should be related to the substrate concentration by a simple Michaelis–Menten equation. However, Winter & Christensen (1964), using a graphical method of analysis, have proposed that the relationship is better expressed by eqn. (10),

$$\text{Flux} = \frac{V.[S]}{K_m+[S]} + D.[S], \tag{10}$$

and they identify the second 'non-saturable' term with a relatively non-specific process of diffusion across the membrane. For leucine fluxes in human erythrocytes at 37° C, they find that the non-saturable pathway comprises 26% of the total flux at low leucine concentrations ([Leu] $\ll K_m$).

In this study, equation (10) was fitted to the data by an alternative technique (D. G. Hoare, manuscript in preparation) in which a computer was used to obtain the best set of values for V, K_m and D to fit each set of data, and also their errors of estimate. Concentrations of leucine in excess of 20 mM were not used, since higher concentrations of leucine must necessarily have a significant effect on the ionic strength if constant osmotic pressure is to be maintained. Within this concentration range, it was not possible to obtain statistically significant values for D at 37° C.

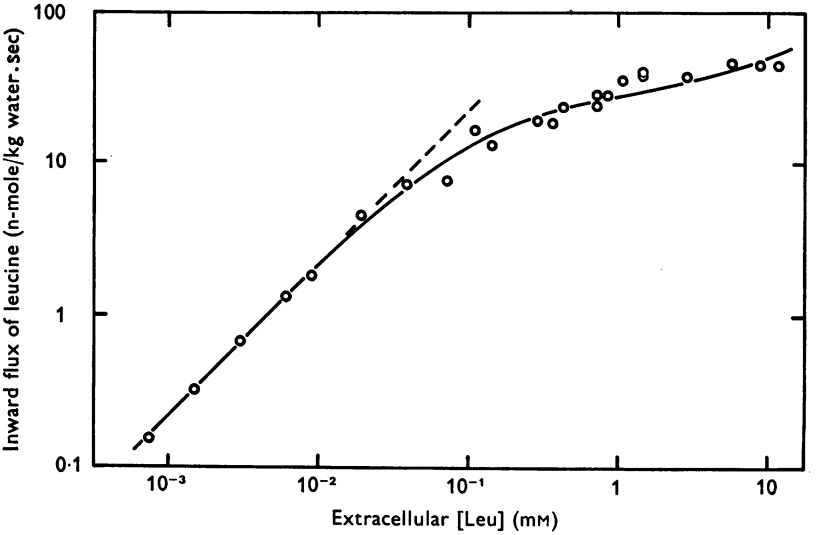

Fig. 4. The relationship between inward leucine flux and extracellular leucine concentration at 2·5° C. The curve is computed from eqn. (10) using the constants in Table 2.

TABLE 2. Values for the parameters of eqn. (10) found on fitting this equation to entry and exit experiments at 2·5° C

V	Entry experiment	Exit experiment
μmole/kg cell water.sec	0·031	0·35
K_m mM	0·13	1·92
D sec^{-1} (with S.E. of estimate)	$(2 \pm 0{\cdot}8) \times 10^{-6}$	$(-5 \pm 13) \times 10^{-6}$

However, the value of K_m for entry experiments decreases rapidly with temperature, and at 2·5° C reaches about 0·3 mM; therefore, under these conditions a leucine concentration of 20 mM exceeds K_m by a factor of about 60, and the non-saturable pathway should constitute a much larger proportion of the total flux. The experimental results for the entry experiment under these conditions are shown in Fig. 4, and the values of

V, K_m and D for both entry and exit experiments are shown in Table 2. Although the value of D found in the transport experiment does appear to be just statistically significant, it corresponds to a non-saturable component that contributes no more than 1 % to the total flux at low leucine concentrations. Consequently, the possible non-saturable component was taken to be zero in analysing the kinetic data in this paper.

The nature of the substrate-carrier association and dissociation processes

The carrier model in Fig. 3 assumes a simple bimolecular association process between the substrate and carrier, and it is important to establish that this is appropriate for leucine transport in erythrocytes, rather than more elaborate schemes which have also been proposed. In the three principal alternatives that have been proposed, the basic carrier system in Fig. 3 is modified as follows.

(i) Exchange of substrate molecules on the carrier (Britton, 1964). An additional step is proposed by which substrate molecules in solution can exchange with those bound to the carrier without the intermediate formation of free carrier, and more rapidly than the normal dissociation of the substrate from the carrier, as shown in eqn. (11). A process of this type would clearly be important in the interpretation of exchange experiments if substrate dissociation were otherwise rate determining.

$$CS^* + S \rightleftharpoons CS + S^* \tag{11}$$

(ii) The binding and simultaneous transport of two substrate molecules by a single carrier (Jacquez, 1964).

(iii) The catalysis of the rate-determining step of the transport process by the binding of a second molecule of substrate on or near to the carrier.

The kinetic consequences of alternatives (i) and (ii) have been explored by Jacquez (1964), and his results show that at low substrate concentrations ($[S] \ll K_m$), Flux $= A[S] + B[S]^2$ in these experiments, where A and B are constants and A may be equal to zero. It can easily be shown that a similar term in $[S]^2$ arises from alternative (iii).

These squared terms provide a test for the validity of these mechanisms. If $\log_{10}$ (flux) is plotted against $\log_{10}[S]$, as is shown in Fig. 5, then at low concentrations ($[S] \ll K_m$) the graph should tend toward a straight line of slope 1·0 if the relationship is given by a simple Michaelis–Menten equation. The presence of a term in $[S]^2$ should increase the gradient, to a value as high as 2·0 if $B \gg A$ in the above equation. However, the data in Fig. 5 show that the gradient does not significantly exceed 1·0, and so none of the three above alternative mechanisms are applicable in this case.

These data also show that at low concentrations of leucine the fluxes for entry and exchange experiments become equal; this is consistent with the expressions for V and K_m in Table 1, since at low substrate concentrations *flux* $\propto V/K_m$, and also arises from Ussing's flux ratio criterion (Ussing, 1949) when the carrier is not near saturation.

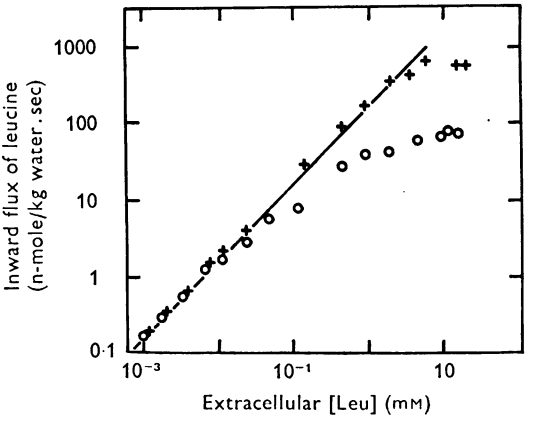

Fig. 5. The relationship between leucine flux and concentration at 5° C. ○, entry experiments; +, exchange experiments. The axes are calibrated on a log scale, and the line has a gradient 1·0.

The possibility of two or more parallel carrier systems

In this paper, considerable emphasis is laid on the kinetic differences between exchange fluxes on the one hand, and entry and exit fluxes on the other. It is therefore important to consider whether these differences could arise from the presence of more than one type of carrier system in the membrane, acting in parallel.

Carrier systems based on that in Fig. 3 can differ considerably in the relationships between exchange and net fluxes. If the re-orientation of the empty carrier is much faster than that of the carrier-substrate complex, then the exchange fluxes tend to be comparable to or slightly slower than the net fluxes. Alternatively, if the re-orientation of the empty carrier is by far the slower step, the carrier system can maintain a rapid exchange flux with a very slow net flux.

The best test for the presence of dissimilar carrier systems of this type in the membrane is to compare exchange and net fluxes at low substrate concentrations ($[S] \ll K_m$). If several independent carrier systems are present, there is no reason to expect the exchange and net fluxes to become equal at low substrate concentrations, since some carriers will be available to exchange transport which may support only a negligible net transport.

On the other hand, if only a single carrier system is providing both the exchange and net fluxes, then the exchange and net fluxes must become equal at low concentrations. This was discussed at the end of the preceding section, and can be seen from the identical expressions for V/K_m which are obtained for entry, exit and exchange reactions from the expressions in Table 1.

The experimental comparison of entry and exchange reactions shown in Fig. 5 shows that the entry and exchange fluxes do become equal at low concentrations. This therefore supports the conclusion that a single carrier system mediates both the exchange and net fluxes of leucine in the human erythrocyte.

Further support for this view comes from inspection of the data on the exchange flux in Fig. 5. If the total exchange flux is in fact the sum of the transport flux shown ($K_m \simeq 0{\cdot}3$ mM), and a specifically exchange carrier of higher K_m, the relationship between log (exchange flux) and log [Leu] could scarcely be a straight line of gradient one up to a leucine concentration as high as 2 mM. Yet this relationship is exactly what is observed.

In conclusion, it seems very unlikely that the differences observed between exchange and net fluxes observed in this system arise from the presence of different types of leucine carrier in the membrane.

The identity of the rate-determining step

By comparing the values of V for entry and exchange experiments it is possible to establish definitely whether the rate-determining step is the association–dissociation processes of the carrier-substrate complex, or the carrier re-orientation processes. Using the information in Table 1, expressions for the ratio of V (*entry*) to V (*exchange*) can be obtained, which are shown below.

Rate-determining step: expression for V (*entry*)/V (*exchange*).

Carrier re-orientation

$$\frac{(1+q/r^2)(1+q)}{pq(1+q/r^2)+(1+q)} = W. \tag{12}$$

Association–dissociation

$$(1+qs/r^2) = X. \tag{13}$$

Clearly, W can adopt any positive value if the appropriate values of p, q, and r^2 are chosen. However, since p, q, and r^2 and s are by definition positive numbers, the value of X must always be greater than or equal to one, and the value of V (*entry*) must, in this case, always be greater than or equal to that of V (*exchange*). Therefore, if the experimental value of V (*entry*) is significantly less than V (*exchange*), the association–dissocia-

tion process cannot be rate-determining. This criterion was also used by Britton (1964), but with the assumption that $q = r^2 = 1$.

Table 3 shows values of V for entry, exit and exchange experiments at 25° C (the values for the entry and exchange experiments are interpolated from data given by Hoare, 1972). The value for V (*entry*) is in fact considerably lower than the corresponding V (*exchange*).

TABLE 3. Values of V (μmole/kg cell water.sec) for entry, exit and exchange experiments at 25° C. V for entry and exchange data are interpolated from more numerous data in the accompanying paper (Hoare, 1972) and have an error of estimate of about $\pm 10\%$. V for exit is the mean of two determinations; an approximate error of estimate is shown

Entry	Exchange	Exit
10·5	21·9	$11{\cdot}2 \pm 0{\cdot}2$

It appears, therefore, that the association–dissociation processes of the carrier-substrate complex cannot be rate-determining, and that the system can be analysed further on the assumption that it is the carrier re-orientation processes which are rate-determining.

The effect of the presence of bound substrate on the carrier re-orientation rate

It has frequently been observed that the substrate flux in exchange experiments is greater than in entry experiments, and this has been attributed to the fact that the carrier-substrate complex can re-orientate more rapidly than the empty carrier (Regan & Morgan, 1964; Levine, Oxender & Stein, 1965; Levine & Stein, 1966; Kotyk, 1967). However, in the general case where q and r^2 are not equal to 1·0, this is an inadequate criterion for establishing a difference in re-orientation rates. The ratio of V (*exchange*) to V (*entry*) (given as W in eqn. (12)) is a function not only of p, but also q and r^2, and so an independent value of p cannot be obtained directly from W. Indeed, even with p equal to one, W can differ considerably from one if the carrier system is highly asymmetric, with q and r^2 significantly different from unity. The same arguments apply to the ratio of V values for exit and exchange experiments which can also be calculated from the expressions in Table 1 and is shown below.

$$V\,(exit)/V\,(exch.) = \frac{(1+q/r^2)(1+q)}{p(1+q/r^2)+(1+q).q/r^2} = Y. \qquad (14)$$

However, an independent value of p can be obtained if experimental values for both W and Y are available. It can easily be shown that

$$1/W + 1/Y = 1+p \qquad (15)$$

and this equation can be used to calculate values for p.

Table 3 contains the required values of V for transport, exit and exchange experiments at 25° C, and the value of p calculated from eqn. (15) is $3{\cdot}05 \pm 0{\cdot}35$.

The distribution of the carrier between the two membrane surfaces at equilibrium

A complete picture of the properties of the carrier system also requires values for q, which describes the distribution of the carrier between the two membrane surfaces at equilibrium, and r^2, which is the ratio of the substrate dissociation constants at the two surfaces. If the calculated value

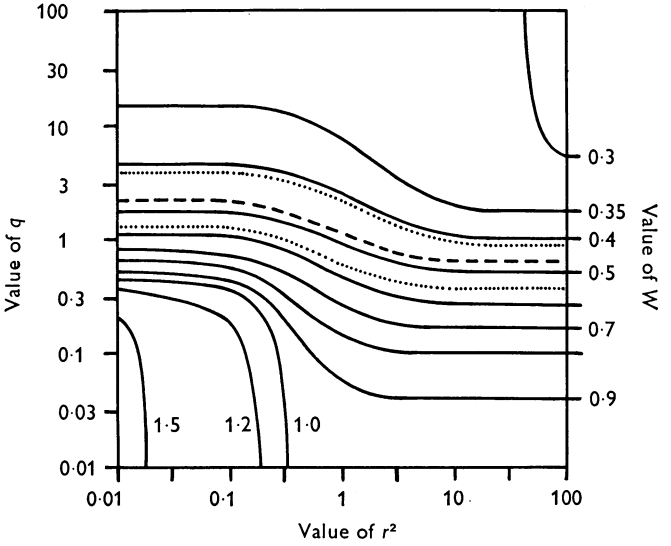

Fig. 6. The relationship between W, q and r^2. Values of q and r^2 are shown on log scales as ordinates and abscissae respectively. Values of W were calculated from eqn. (12), with $p = 3{\cdot}05$ (the experimental value for 25° C), and were used to construct the contours shown in the diagram, from which the W-value corresponding to any pair of q and r^2 values can be interpolated. See text for further discussion.

for p, and the experimental value for W are substituted into eqn. (12), the two unknowns q and r^2 remain, and there is in principle no general way of obtaining independent values for either. The fact that the carrier system exhibits an over-all functional symmetry at 37° C (Winter & Christensen, 1964) does not establish a unique value for either q or r^2. In practice, however, approximate values for q can be obtained.

Fig. 6 shows values of W calculated from eqn. (12), using the value of p derived above (3·05) and a range of values for q and r^2. The Figure shows that the calculated value of W depends markedly on the value of q chosen,

but is much less dependent on the value of r^2. The broken line in Fig. 6 corresponds to the experimental value for W at 25° C, and it is clear that for this value of W, q must lie in the range 0·7–1·9 whatever value for r^2 is adopted. Naturally, the experimental value of W used will be subject to an error of estimate; this is hard to assess accurately, but if the interpolated values of V (*exchange*) and V (*entry*), whose ratio gives W, are assigned a reasonable error of estimate of $\pm 10\%$, the over-all error of estimate in W is about $\pm 14\%$. These limits are marked in as dotted lines in Fig. 6. The effect of this experimental error of estimate is to extend the range of possible values of q to 0·3–4·5.

In conclusion, the value for the constant q describing the equilibrium distribution of the carrier between the inner and outer membrane surfaces depends on the value adopted for r^2, the ratio of dissociation constants. However, q is defined to within about an order of magnitude regardless of what value of r^2 is assumed.

DISCUSSION

The interpretation of the kinetics of carrier transport have always rested rather heavily on the assumption that it is the carrier re-orientation steps that are rate-determining, although Dawson & Widdas (1964) have proposed that at low temperatures the dissociation of glucose from the carrier may be rate-limiting, and this assumption is also implicit in the suggestion that exchange processes on the carrier surface (eqn. (11) above) may affect flux rates (Britton, 1964). It is therefore reassuring that the criterion previously used to test whether carrier re-orientation was rate-determining (Britton, 1964) is in fact also valid for the more general case, and that the carrier re-orientation can be definitely be identified as the rate-determining step in leucine transport.

The situation on the relative rates of re-orientation of substrate-carrier complex and empty carrier has also been clarified. From a general viewpoint, some doubt must hang over previous re-orientation rate ratios determined for the glucose carrier since these could have arisen either from a genuine difference in the rate constants, or from a kinetic asymmetry of the carrier system with respect to the inner and outer surface of the membrane. As far as the leucine carrier is concerned, the previous data on a methionine-induced counterflux of leucine (Winter & Christensen, 1964) could have arisen from different re-orientation rates, or could simply have arisen through competitive inhibition of the inward flux of leucine by the extracellular methionine (Rosenberg & Wilbrant, 1957). However, it has now proved possible to show that the exchange flux is not accelerated by an exchange process on the carrier surface as shown in eqn. (11) (Britton, 1964), and to obtain unambiguous values for the re-orientation

rate ratio, p. Despite the doubt which attaches to earlier values of this ratio for the glucose carrier, it is reassuring that the value for p for the leucine carrier at 25° C ($p = 3{\cdot}05$) is of the same order of magnitude as the values found for the glucose carriers (Levine & Stein, 1966; Mawe & Hempling, 1965).

The approximate value for q given above constitutes the first information published on the equilibrium distribution of a carrier. Previously, there has been no information to eliminate highly asymmetric mechanisms in which the carrier operates almost entirely from a preferred inward or outward orientation. The analysis used in this paper cannot give a precise value for the distribution constant, q, but it does eliminate some of the more highly asymmetric possibilities, and indicates that at 25° C the equilibrium distribution of carrier is approximately equal between inward and outward orientations. Although it is too early to relate the carrier distribution between the two membrane surfaces to the features of the membrane structure, this will clearly be an interesting problem when more details of membrane structure are known.

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