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THE TEMPERATURE DEPENDENCE OF THE TRANSPORT OF L-LEUCINE IN HUMAN ERYTHROCYTES

By D. G. HOARE

From the Department of Biology, University of York, Heslington, York

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SUMMARY

1. Experiments are reported on the entry, exit and exchange fluxes of L-leucine in human erythrocytes in the temperature range 0–37° C. The kinetics of entry and exit become quite different at low temperatures, indicating an asymmetry in the transport mechanism.

2. The data is analysed in terms of a simple carrier mechanism. This shows that carrier re-orientation is rate determining at all temperatures studied, and suggests, although does not prove, that the origin of the asymmetry of the carrier may be an unequal equilibrium distribution of the carriers between inward and outward orientations.

3. The activation energy increases sharply on going to low temperatures. This increase is the sum of two components. One arises from differences between the activation energies for inward and outward carrier re-orientations, and the other from changes, with temperature, in the activation energies of the individual carrier re-orientation steps.

INTRODUCTION

Studies of the temperature dependence of biological transport processes can be of great interest since they provide information on the activation energies of these processes, and particularly on the way in which these activation energies change with temperature. These parameters can provide criteria for detecting changes in rate determining step in the transport process, and ultimately for judging the suitability of possible molecular mechanisms.

Studies on the temperature dependence of glucose transport in human erythrocytes (Sen & Widdas, 1962; Dawson & Widdas, 1964; Bolis, Luly, Pethica & Wilbrandt, 1970) have led to several suggestions on how changes in activation energy can be explained in terms of simple carrier mechanisms. The difficulty in deciding between these possibilities

emphasizes the importance of attributing activation energy changes, as far as possible, to individual steps in the carrier mechanism.

In this paper, data from experiments on the entry, exit and exchange of L-leucine in human erythrocytes are interpreted in terms of a new kinetic analysis (Hoare, 1972, the preceding paper) based on the simple carrier model shown in fig. 3 of the preceding paper. This kinetic analysis is based on the use of dimensionless ratios of rate constants in the kinetic equations which are summarized in the Methods section.

The principal experimental finding in this paper is a very marked increase in the activation energy for the leucine transport at low temperatures; at higher temperatures, the results are generally consistent with those of Winter & Christensen (1964) in the 27–37° C range, who found a Q_{10} of about 1·7. Comparable increases in activation energy have been observed for β-galactoside transport in *E. coli* (Kotyk, 1967) and glucose transport in human erythrocytes (Sen & Widdas, 1962; Dawson & Widdas, 1964; Bolis *et al.* 1970), but the maximum activation energies reported are lower than that found for leucine transport in this paper. It is possible to show that the explanations offered to account for the changes in activation energy observed with glucose transport cannot account for the changes observed for the leucine system. These must arise from the asymmetry of the carrier system between the two surfaces of the membrane, or from changes in the mechanism of the carrier re-orientation process itself.

METHODS

The experimental methods and materials used have been described in detail in the preceding paper (Hoare, 1972).

Three types of kinetic experiments were conducted. *Entry experiments* involved measuring the inward flux of L-leucine into erythrocytes which had been previously washed free of intracellular leucine, and *exit experiments* involved measuring the outward flux from erythrocytes preloaded with leucine into a leucine-free medium. In *exchange experiments*, the erythrocytes were first allowed to come to equilibrium in a solution of non-radioactive leucine; since leucine transport is non-concentrative in human erythrocytes (Winter & Christensen, 1964), this corresponded to the intracellular and extracellular concentrations being equal. The inward flux was then measured by adding [^{14}C]leucine to the extracellular solution.

Since the relationship between leucine flux and concentration in these experiments is satisfactorily given by initial flux $= V.[\text{Leu}]/(K_m + [\text{Leu}])$ (Hoare, 1972), values of V, K_m and V/K_m for all three types of experiments could be obtained by plotting 1/(initial flux) *vs.* 1/[Leu]. A straight line was fitted to the data using a weighted linear regression.

The kinetic analysis in the preceding paper is based on using suitably defined dimensionless ratios of rate constants to simplify the kinetic equations. This focuses attention on the particular properties of the carrier system, such as the asymmetry in the equilibrium distribution of carriers, which these ratios represent. These ratios are defined as follows in terms of the rate constants in Fig. 3 of the preceding paper (Hoare, 1972).

Symbols used in kinetic analysis

The symbols used for the kinetic analysis of the carrier model of Fig. 3 in the previous paper are also used in this paper with the following additional symbols:

CS	carrier–substrate complex,
*E**	Arrhenius activation energy,
ΔH	enthalpy change,
exch.	exchange experiment.

Subscripts used have also the same significance as in the previous paper.

Mean re-orientation rates, and the effect of substrate binding on the carrier re-orientation rate. Since inward and outward carrier re-orientation rates may not be equal, mean rate constants can be defined as follows:

$$2/k_s = 1/k_{si} + 1/k_{so}, \tag{1}$$

$$2/k_c = 1/k_{ci} + 1/k_{co}. \tag{2}$$

The effect of substrate binding on the re-orientation rate can then be expressed by a ratio of rate constants, p:

$$p = k_s/k_c. \tag{3}$$

Mean dissociation constants. The dissociation constants of the substrate from the carrier on the inner surface (K_{si}) may not equal that on the outer surface (K_{so}). Therefore a mean and a ratio of the two are defined as follows:

$$K_s^2 = K_{si}.K_{so} = (k_{di}/k_{bi}).(k_{do}/k_{bo}), \tag{4}$$

$$r^2 = k_{si}/k_{so}. \tag{5}$$

Asymmetry of the carrier re-orientation rates. The ratio between the inward and outward re-orientation rate constants for the empty carrier is designated q, which is, of course, also the equilibrium constant for the distribution of the empty carrier between the two membrane surfaces

$$q = k_{ci}/k_{co}. \tag{6}$$

Reference to Fig. 3 of the preceding paper (Hoare, 1972) shows that the equilibrium constant for the distribution of the carrier–substrate complex between the two membrane surfaces must be given by eqn. (7):

$$k_{si}/k_{so} = q.K_{so}/K_{si} = q/r^2. \tag{7}$$

Units of energy. In this paper, kJ have been used as units of energy, to conform to the SI units. These are related to the more familiar kcal by 1·0 kcal = 4·18 kJ.

RESULTS

It was shown in the preceding paper (Hoare, 1972) that the relationship between leucine flux and concentration can be expressed by a Michaelis–Menten equation such as eqn. (8):

$$\text{Initial flux} = V.[\text{Leu}]/(K_m + [\text{Leu}]). \tag{8}$$

This applied equally to entry, exit and exchange experiments (defined in Methods section). However, although the same form of equation relates

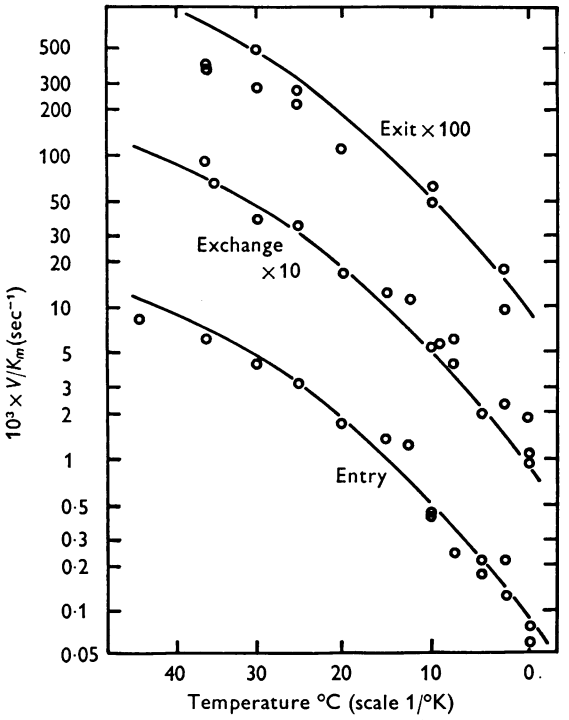

Fig. 1. The temperature dependence of V/K_m for entry, exit and exchange experiments. For clarity, the values for exchange and exit are displaced vertically by multiplying by 10 and 100 respectively. Identical curves are drawn through each set of data for comparison.

flux and concentration in the two types of experiments, this does not necessarily imply that V and K_m have the same numerical values in entry, exit and exchange experiments. Different expressions for V and K_m are obtained for the three types of experiments when they are expressed in terms of the means and ratios of rate constants defined in fig. 3 of the preceding paper and eqns. (1)–(7) (Hoare, 1972). The experimental results given below show that different values of V and K_m are obtained from the different types of experiments.

In contrast, an important feature of these expressions, which is inherent in any carrier model not involving active transport, is that V/K_m should be equal for entry, exit and exchange reactions. In fact, V/K_m is simply the rate constant governing facilitated diffusion of substrate across the membrane at low concentrations when the carrier is far from saturation. This can readily be demonstrated for the general carrier model in Fig. 3 of the preceding paper, using the expressions for V and K_m derived also in the preceding paper (Hoare, 1972). The experimental values for V/K_m

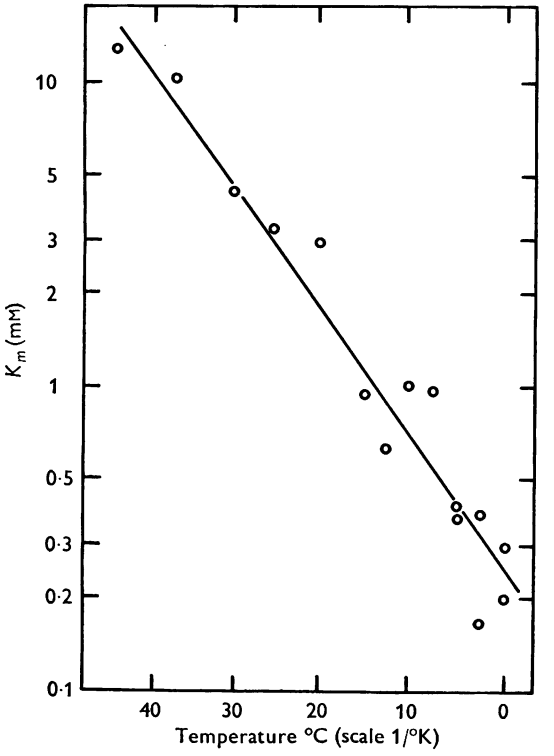

Fig. 2. The temperature dependence of K_m for entry experiments.

obtained over a wide temperature range are shown in Fig. 1. The values for entry, exit and exchange reactions are separated by a vertical displacement for clarity, but they are fitted by identical curves. These data bear out the prediction that V/K_m should be equal for the three types of experiment. However, they show an additional feature of interest; the gradient of the curve increases considerably at low temperatures. Since the scales of the ordinates and abscissae are equivalent to a graph of log (*flux*) *vs.* 1/(*temperature*), the gradient is proportional to the Arrhenius activation energy, E^*, and the change in gradient indicates an increase in activation energy at low temperatures.

Temperature dependence of V and K_m

Since V/K_m is a composite rate constant, the activation energy associated with it is the sum of the corresponding energy terms associated with V and $1/K_m$ individually; the change in its value with temperature could arise from a change in either or both of these components. Figs. 2 and 3 show the temperature dependence of K_m values for entry, exchange and exit experiments plotted in the same manner. In all cases the points

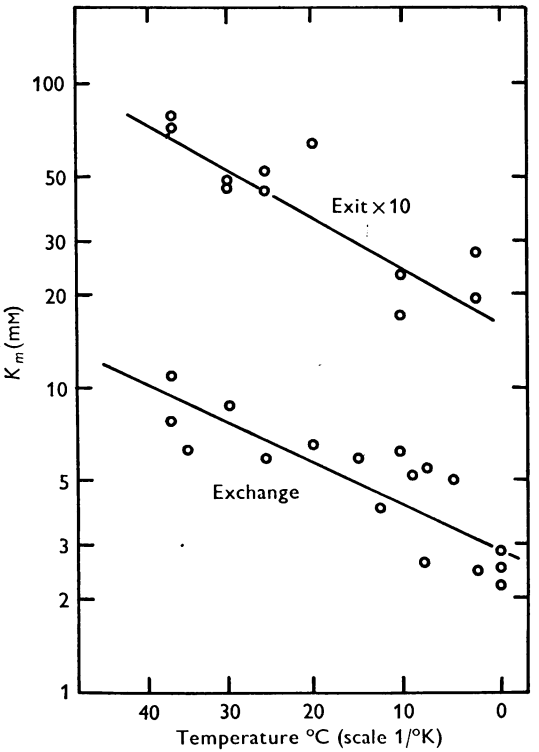

Fig. 3. The temperature dependence of K_m for exchange and exit experiments. For clarity, the data on exit experiments is displaced vertically by multiplying by 10.

lie on an approximate straight line and certainly show no evidence for a sharp change in gradient. However, the data in Figs. 4 and 5, which show the temperature dependence of V for entry, exchange and exit experiments, indicate that there is a marked change in the activation energy associated with V over the temperature range. At least qualitatively, then, it appears that the change in activation energy associated with V/K_m arises from the V term rather than the K_m term.

The rate-determining step in leucine transport

Hoare (1972) has shown that the dissociation of leucine from the carrier cannot be rate-limiting so long as $V(entry)$ is less than $V(exch)$, and this was shown to be true at 25° C. The data in Figs. 4 and 5 show that $V(entry)$ is smaller than $V(exch)$ over the entire temperature range 2·5–37° C, and therefore the substrate dissociation step cannot be rate-determining under any of the conditions discussed in this paper.

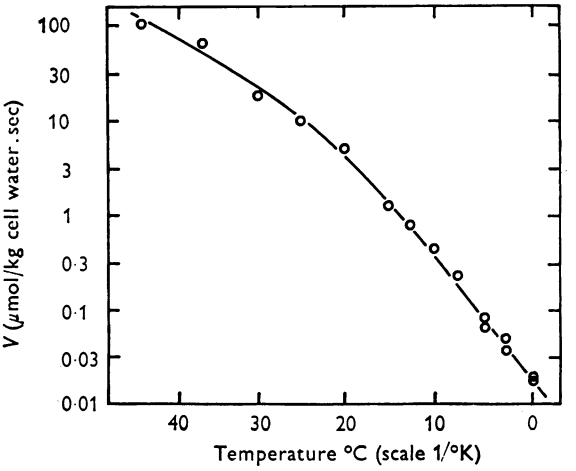

Fig. 4. The temperature dependence of $V(entry)$. The curve is derived from eqn. (10) by a curve-fitting procedure discussed in the text.

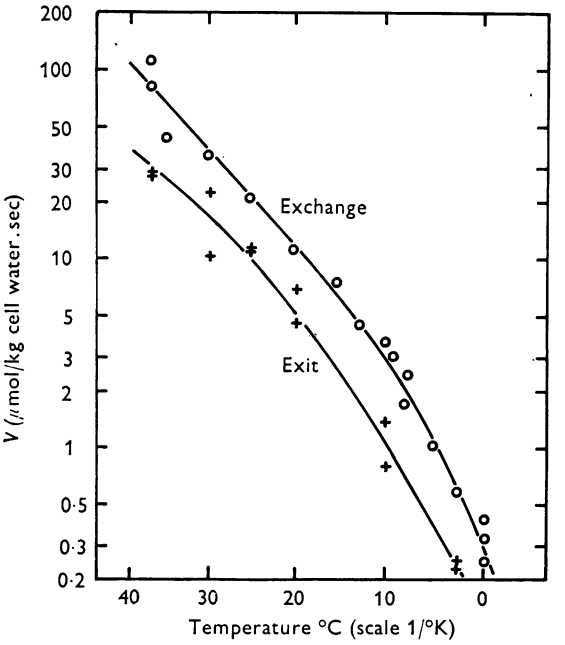

Fig. 5. The temperature dependence of $V(exch)$ and $V(exit)$. The curve for $V(exch)$ is derived from eqn. (14) by a curve-fitting procedure discussed in the text.

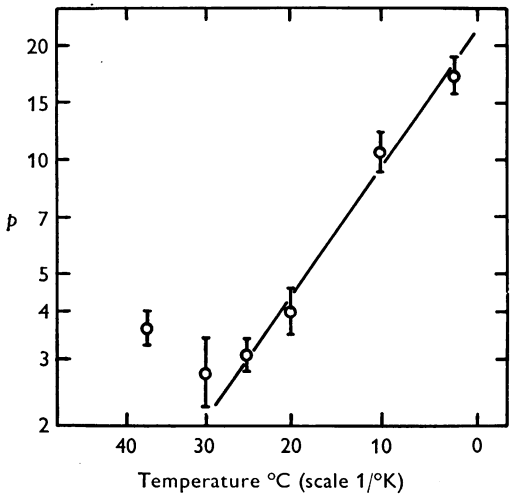

Fig. 6. The temperature dependence of p, the ratio of mean re-orientation rate constants of the loaded and empty carriers. p is calculated from eqn. (9). Values of $V(entry)$ and $V(exch)$ are obtained by interpolation from the fitted curves in Figs. 4 and 5 and assigned an error of estimate of $\pm 10\%$. Values of $V(exit)$ are taken from Fig. 5 and assigned an error of estimate equal to the mean error of the duplicate determinations.

The effect of the binding of substrate on the carrier re-orientation rate

An interesting feature of leucine transport in human erythrocytes is that the leucine–carrier complex can re-orientate considerably more rapidly than the empty carrier. This can be expressed quantitatively by the ratio p, which was defined in eqns. (1)–(3) as the ratio of the mean re-orientation rate of the leucine–carrier complex to the mean re-orientation rate of the empty carrier. Hoare (1972) has shown in the preceding paper that p can be derived from the value of V for entry, exit and exchange experiments using eqn. (9),

$$p+1 = V(exch)\,(1/V(entry)+1/V(exit)). \qquad (9)$$

Fig. 6 shows the values of p over a temperature range, derived from the values of V in Figs. 4 and 5. These results reveal a very interesting feature of the leucine transport system. While the value of p does not alter dramatically in the temperature range 20–37° C, it increases very sharply below 20° C, reaching a value exceeding 15 at 0° C. Some change in the transport system must occur at or below 20° C which makes the membrane discriminate increasingly sharply between the leucine–carrier complex and the empty carrier at low temperatures.

The asymmetry of the carrier system

A carrier system is asymmetric if the kinetics of the substrate entry process differ from the kinetics of the exit process. An asymmetry of this kind can arise from an unequal distribution of the carrier between the two membrane surfaces (given quantitatively by the equilibrium constant, q, defined in eqn. (6)), or from differences between the dissociation constant of the carrier–substrate complex at the two membrane surfaces (defined by the dissociation constant ratio, r^2, defined in eqns. (4) and (5)).

The data on V and K_m in Figs. 2–5 show that, even though the carrier appears to be functionally symmetrical around room temperature, it becomes highly asymmetrical at low temperatures. Under these conditions, V and K_m for the entry process are considerably smaller than V and K_m for the exit experiment.

It is not possible to diagnose with complete certainty whether this kinetic asymmetry arises from unequal distribution of the carrier, or from changes in dissociation constant, between the two membrane surfaces. However, Hoare (1972) has described a procedure by which q can be determined approximately, and the values of q calculated in this way are shown in Fig. 7.

Two factors govern the precision with which q can be determined. The first is the ratio of dissociation constants, r^2. One cannot determine q precisely without knowing the value of r^2, and these experiments provide no way of evaluating r^2. However, q can be limited to a definite range of values, whatever value r^2 has between $+\infty$ and zero (r^2 cannot by definition adopt negative values); these ranges are represented by the continuous bars in Fig. 7. In addition to the uncertainty about the value of r^2, there is an experimental error in the values of V used to determine q (this introduces an error of estimate in W – cf. eqn. (12) of the preceding paper, Hoare, 1972). Consequently, the error of estimate of q is extended by the amount represented by the dashed portion of the bars in Figure 7.

Without some independent way of determining r^2, which would reduce the uncertainty in the value of q to the length of the dashed portion of the bars, the data in Fig. 7 lacks sufficient accuracy to allow any very definite conclusions to be drawn about the effect of temperature on the distribution of the carriers between the membrane surfaces. However, the results certainly suggest that the carrier tends to concentrate at the inner membrane surface at low temperatures, and that this may be the origin of the kinetic asymmetry observed in leucine transport under these conditions.

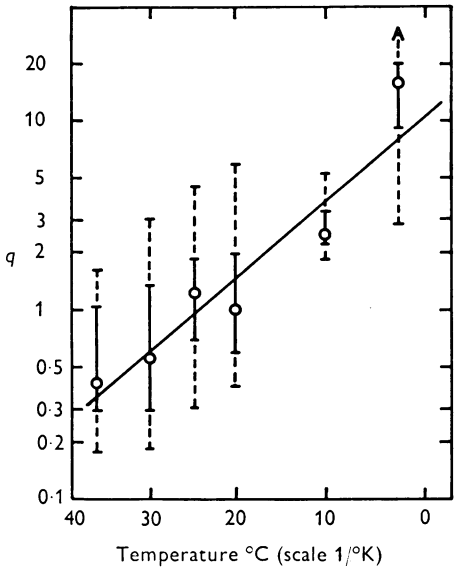

Fig. 7. The temperature dependence of q, the equilibrium constant for the distribution of carriers between inward and outward orientation. q is evaluated by the procedure of Hoare (1972). ○, values of q calculated on the assumption that the dissociation constant ratio, r^2, equals 1; solid bars, range of possible q values if r^2 ranges from $+\infty$ to 0; dashed bars, maximum range of q values allowing also for experimental errors in determinations of rate constants.

Quantitative evaluation of activation energies

The temperature dependencies of V and K_m shown in Figs. 2–5 suggested that the apparent change in activation energy for the transport process is associated with the V term, rather than the K_m term, in the Michaelis–Menten equation. Before this information can yield any valuable insight into the nature of the mechanism of action of the carrier, one must first measure the values of the apparent activation energies E^* (*Ventry*) and E^* (*Vexch*) from the slopes of the curves relating V to temperature, and secondly, identify these activation energies with particular steps in the carrier mechanism.

Experimental values for the activation energies associated with $V(entry)$ and $V(exch)$ (viz. E^* (*Ventry*) and E^* (*Vexch*) respectively), were derived from the data in Figs. 4 and 5 by a curve-fitting procedure. The empirical function chosen to fit the data is shown in eqn. (10); it was chosen because the relationship between V and T which it embodies appears closely to parallel that observed experimentally.

$$1/V = \exp(A/T - B) + \exp(C/T - D). \qquad (10)$$

TABLE 1. Apparent Arrhenius activation energies. $E^*(Ventry)$ and $E^*(Vexch)$ are derived respectively from V for entry and exchange experiments by the curve-fitting procedure described in the text. E^*_{kc} and E^*_{ks} are related to the mean carrier re-orientation rate constants k_c and k_s, and are derived from $E^*(Ventry)$ and $E^*(Vexch)$ by eqns. (17) and (18) (see also text)

Temperature (° C)	Confidence limit	$E^*(Ventry)$ (kJ/mol)	$E^*(Vexch)$ (kJ/mol)	E^*_{kc} (kJ/mol)	E^*_{ks} (kJ/mol)
	−95%	41	—	—	—
	−75%	50	25	—	—
37	Best value	71	59	94	59
	+75%	100	80	—	—
	+95%	109	88	—	—
	−95%	169	126	—	—
	−75%	174	141	—	—
0	Best value	203	197	212	197
	+75%	227	307	—	—
	+95%	248	—	—	—

Since $E^* = -2{\cdot}3R.\mathrm{d}(\log V)/\mathrm{d}(1/T)$, the relationship between E^* and the parameters of the empirical function can be obtained by differentiating eqn. (10) with respect to $(1/T)$, rearranging and substituting for E^*; this yields eqn. (11):

$$E^* = V.R.(A.\exp(A/T-B)+C.\exp(C/T-D)). \qquad (11)$$

The curve-fitting procedure involved use of a digital computer, and for each set of data produced an approximate best set of values of A, B, C and D, with confidence limits (D. G. Hoare, manuscript in preparation). These values were used to compute the curves shown in Figs. 4 and 5 (exchange experiment only). The activation energies at 0° C and 37° C were then calculated using eqn. (11), and approximate errors of estimate obtained by altering the set of values of A, B, C and D within the appropriate confidence limits.

The results are shown in Table 1. For both transport and exchange experiments, the activation energy reaches very high values at 0° C, and changes considerably over the experimental temperature range. The increase in activation energy on going from 37 to 0° C can be estimated as 132 ± 34 kJ/mole for transport experiments, and 138 ± 51 kJ/mole for exchange experiments.

The activation energies associated with the mean re-orientation rate constants

Even in a relatively simple carrier model such as that in Fig. 3 of the preceding paper, there are many independent rate constants, each of which may have a different temperature dependence. There is, therefore, no

difficulty in accounting qualitatively for a change with temperature in the observed activation energy for the transport process. Two such suggestions for the glucose transport system in erythrocytes have been made by Dawson & Widdas (1964) and Bolis *et al.* (1970), both of which suggest a change in rate-determining step. The number of possibilities can be limited if the overall activation energy for the transport process can be used to deduce, as far as possible, the activation energies associated with individual steps in the carrier mechanism. In this section, the experimental values of the overall activation energy, E^* (*Ventry*) and E^* (*Vexch*) are used to calculate the activation energies associated with the mean re-orientation rate constants, $E^*_{k_s}$ and $E^*_{k_c}$ (the mean re-orientation rate constants, k_s and k_c, are defined in eqns. (1) and (2)).

It was shown in the preceding paper (Hoare, 1972) that the expressions for V (*entry*) and V(*exch*) are given by eqns. (12) and (13):

$$V(exch) = k_s.\{C\}/2 \tag{12}$$

$$V(entry) = \frac{k_s.\{C\}}{2}.\frac{(1+q/r^2)(1+q)}{pq(1+q/r^2)+(1+q)}. \tag{13}$$

Combining eqn. (13) with eqn. (3) gives eqn. (14):

$$V(entry) = \frac{k_c.\{C\}}{2}.\frac{p(1+q/r^2)(1+q)}{pq(1+q/r^2)+(1+q)}. \tag{14}$$

Dividing eqn. (12) by eqn. (13) gives

$$\frac{V(entry)}{V(exit)} = \frac{(1+q/r^2)(1+q)}{pq(1+q/r^2)+(1+q)}. \tag{15}$$

Eqns. (9) and (15) can now be used to eliminate p, q and r^2 from eqn. (13), yielding

$$\frac{k_c.\{C\}}{2} = \frac{V(entry)}{\left\{1+\frac{V(entry)}{V(exit)}-\frac{V(entry)}{V(exch)}\right\}}. \tag{16}$$

Eqns. (12) and (16) can now be used to determine $E^*_{k_s}$ and $E^*_{k_c}$.

$E^*_{k_s}$ can be obtained by taking logarithms through eqn. (12), differentiating with respect to $1/T$ and substituting

$$E^*_{k_s} = -2{\cdot}3R.\,\mathrm{d}(\log k_s)/\mathrm{d}(1/T)$$

and

$$E^*\,(Vexch) = -2{\cdot}3R.\,\mathrm{d}(\log V(exch))/\mathrm{d}(1/T).$$

This yields

$$E^*_{k_s} = E^*\,(Vexch) \tag{17}$$

and $E^*_{k_s}$ can be equated directly to the experimentally determined E^* (*Vexch*).

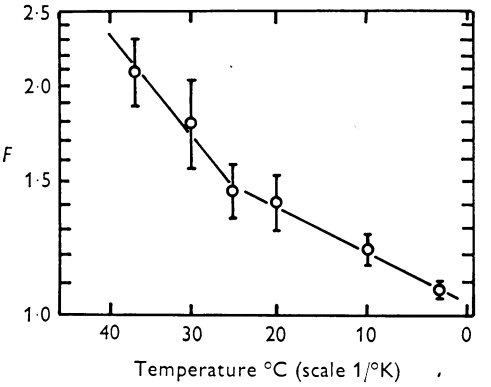

Fig. 8. The temperature dependence of the function

$$F = 1 + V(entry)/V(exit) - V(entry)/V(exch),$$

required in text for eqns. (16) and (18). Values of $V(entry)$, $V(exit)$ and $V(exch)$ are taken from Figs. 4 and 5; errors of estimate are discussed in caption for Fig. 6. The data is fitted by two straight lines, for the temperature ranges 2·5–20° C and 20–37° C.

$E^*_{k_c}$ can be obtained by applying a similar process to eqn. (16), which yields

$$E^*_{k_c} = E^*(Ventry) + \frac{2{\cdot}3R.\mathrm{d}(\log[1 + V(entry)/V(exit) - V(entry/V(exch)])}{\mathrm{d}(1/T)}. \tag{18}$$

In order to obtain a value for $E^*_{k_c}$ from E^* ($Ventry$), the second term on the right hand side must be evaluated. This can be done directly from a graph of $\log(1 + V(entry)/V(exit) - V(entry)/V(exch)$ against $1/T$; the gradient of this graph multiplied by $2{\cdot}3R$ gives the required term. Fig. 8 shows this graph calculated from the experimental values of $V(entry)$, $V(exch)$ and $V(exit)$. Calculation from the gradient of this graph shows that $E^*_{k_c}$ exceeds E^* ($Ventry$) by $9{\cdot}2 \pm 3{\cdot}0$ kJ/mol in the range 2·5–20° C and by 23 ± 8 kJ/mole in the range 20–37° C.

The values of $E^*_{k_s}$ and $E^*_{k_c}$ determined above are entered in Table 1.

It can be seen that the large change in the overall activation energy for the transport process arises from changes in $E^*_{k_s}$ and $E^*_{k_c}$. However, k_s and k_c are themselves the means of pairs of rate constants (cf. eqns. (1) and (2)), and therefore the changes in $E^*_{k_s}$ and $E^*_{k_c}$ could arise either from large differences between the activation energies of the individual rate constants, or from changes with temperature of the activation energies associated with these individual rate constants. The balance of evidence supporting these two possibilities is evaluated in the Discussion.

DISCUSSION

The results described in this paper raise two questions of particular interest. First, what is the origin of the large change in activation observed experimentally in leucine transport into erythrocytes, and secondly, why is the leucine bound to the carrier able to accelerate its re-orientation much more effectively at low temperatures than at high temperatures? Although these questions must ultimately be answered in terms of molecular mechanisms, the evidence available at present does not allow one to go beyond a purely kinetic analysis in terms of the carrier mechanism considered in this paper.

The origin of the change in activation energy

The data in this paper allows one to calculate values for E^*_{ks} and E^*_{ks}, the activation energies associated with the *mean* re-orientation rate constants, k_s and k_c, defined in eqns. (1) and (2). This is still one step removed from the ultimate aim of deriving activation energies for *individual* carrier re-orientation steps.

The relationships of the mean re-orientation rate constants to the individual rate constants are given in eqns. (19)–(22), which are derived from eqns. (1), (2), (6) and (7):

$$\tfrac{1}{2}k_c = k_{ci}/(1+q), \tag{19}$$

$$\tfrac{1}{2}k_c = k_{co}.q/(1+q), \tag{20}$$

$$\tfrac{1}{2}k_s = k_{si}/(1+q/r^2), \tag{21}$$

$$\tfrac{1}{2}k_s = k_{so}./(r^2(1+q/r^2)), \tag{22}$$

Taking eqn. (19) as an example, the relationship between $E^*_{k_c}$ and $E^*_{k_{ci}}$ can be obtained by taking logarithms throughout, differentiating with respect to $1/T$, and substituting

$$E^*_{k_c} = -2{\cdot}3R.\,\mathrm{d}(\log k_c)/\mathrm{d}(1/T), \quad E^*_{k_{ci}} = -2{\cdot}3R.\,\mathrm{d}(\log k_{ci})/\mathrm{d}(1/T)$$

and

$$\Delta H_q = -2{\cdot}3R.\,\mathrm{d}(\log q)/\mathrm{d}(1/T).$$

ΔH_q is the enthalpy term associated with re-orientating the empty carrier from the outer to the inner membrane surface and must therefore be equal to the difference of the activation energies for inward and outward carrier re-orientation, $E^*_{k_{ci}} - E^*_{k_{co}}$. The final relationship is shown in eqn. (23):

$$E^*_{k_c} = E^*_{k_{ci}} + \Delta H_q.q/(1+q). \tag{23}$$

Inspection of eqn. (23) shows that the experimentally observed change in $E^*_{k_c}$ could arise from two origins, which are not mutually exclusive. These are as follows.

(*a*) A change in the value of q with temperature. If q changes with temperature, then ΔH_q is not zero. The contribution which the term

$\Delta H_q.q/(1+q)$ makes to E^*_{kc} will vary with temperature, as q changes. It is, of course, possible that ΔH_q itself may also change with temperature.

(*b*) A change in E^*_{kci} with temperature.

These two possibilities are considered in more detail below. Similar treatment of eqns. (20), (21) and (22) lead to other equations similar to eqn. (23).

The contribution of ΔH_q to the activation energy change

If the change in observed activation energy arises from the second term in eqn. (23), $\Delta H_q.q/(1+q)$, then the observed increase in activation energy arises from a change with temperature in the equilibrium distribution of the carrier between the inner and outer surfaces of the membrane. If this is the sole origin of the change in observed activation energy, then it is not necessary to postulate that the activation energy for any of the steps in the carrier mechanism alters with temperature. In this case, of course, ΔH_q is also constant, since $\Delta H_q = E^*_{kci} - E^*_{kco}$.

Since the observed increase in activation energy on going to low temperatures is about 116 kJ/mol (Table 1), then the value of ΔH_q must be at least -116 kJ/mol to account for this change. If ΔH_q is around -116 kJ/mol, then q must change from $q \ll 1$ at 37° C to $q \gg 1$ at 0° C to account for the observed activation energy change. If q is always greater than one, or less than one, throughout the 0–37° C temperature range, then much more negative values of ΔH_q are required to account for the observed changes in activation energy.

A second possibility is that ΔH_q itself changes by around 116 kJ/mol in the experimental temperature range.

The data relating values of q to temperature is not sufficiently precise to allow a value for ΔH_q to be determined. However, the slope and position of the line shown in Fig. 7 is equivalent to $\Delta H_q = -70$ kJ/mol, and a contribution of 35–50 kJ/mole to the observed change in activation energy. This falls far short of the experimentally observed change of 116 kJ/mol, which would require a line of at least double the slope to be drawn through the data. Although this cannot be ruled out, it certainly does not provide the best fit for the data, and one must consider the possibility that a change in the asymmetrical distribution of carriers between the membrane surfaces cannot alone account for observed activation energy changes.

The contribution of E^*_{kci} to the activation energy change

If the observed change in activation energy change is greater than the change in $q.\Delta H_q/(1+q)$ across the experimental temperature range, then eqn. (23) shows that the activation energy for outward carrier re-orientation, E^*_{kci}, must change sufficiently to make up the difference. One can

easily show by differentiating eqn. (20) in the same way as eqn. (19), that an equal change must occur in E^*_{kco} under these circumstances. This would be of particular interest since it would involve a demonstration that the activation energy of *individual* steps in the carrier mechanism undergo marked changes. From this it would follow either that there is some change in mechanism of carrier re-orientation with temperature, or that the carrier re-orientation step itself consists of two successive processes with very different activation energies. Either interpretation would be of great interest since they both bear directly on the molecular mechanism of carrier re-orientation.

If one takes the straight line in Fig. 7 as providing the best fit for the data on the temperature dependence of q, then the contribution of the $q.\Delta H_q/(1+q)$ term in eqn. (23) is 35–50 kJ/mol, and the activation energies for the inward and outward re-orientation steps of the empty carriers must increase by 65–80 kJ/mol on going from 37 to 0° C. The reliance which can be placed on this figure is, however, dominated by the considerable uncertainty about the contribution of the $q.\Delta H_q/(1+q)$ term.

The data in this and the preceding paper (Hoare, 1972) allow a number of conclusions to be drawn about the transport of L-leucine in human erythrocytes. The kinetics of the transport process appear to be consistent with the operation of a simple carrier of the type shown in Fig. 3 of the preceding paper (Hoare, 1972), and it has been possible to eliminate a number of possible alternatives. The dissociation of leucine from the leucine–carrier complex is not the rate-determining step in the temperature range studied (2·5–37° C). At low temperatures, the transport system shows a definite asymmetry, in that the kinetics of the entry process differ markedly from the kinetics of the exit process. The rough data on the distribution of the carrier at equilibrium between the inner and outer membrane surfaces strongly suggests that this may arise from an alteration of this distribution at low temperatures which results in an increase in the proportion of carriers with an inward orientation. The data is not sufficiently accurate to prove this conclusively, and so one cannot rule out the alternative, that the asymmetry may arise from a considerable difference in the dissociation constant of the carrier–substrate complex between the two membrane surfaces at low temperature. However, since the transport process definitely shows kinetic asymmetry, the carrier system must be asymmetric in one or other, if not both, of these ways.

Considerable increases in activation energy on going to low temperatures have been observed for glucose transport in erythrocytes by Dawson & Widdas (1964) and Bolis *et al.* (1970), but the explanations which they offered for these changes in the glucose transport system differ from the conclusions drawn in this paper for the leucine transport system. Both

Dawson & Widdas, and Bolis *et al.* started with a symmetrical carrier model in which q and r^2 (as defined in this paper) are equal to one. Dawson & Widdas suggested that the dissociation of the glucose from the carrier had a very large positive enthalpy, and that this step became rate limiting at low temperatures. Bolis *et al.* concluded that, although this was a possible explanation, the increase in activation energy at low temperature could also arise, with carrier re-orientation rate determining, if the activation energy for re-orientation of the loaded carrier differs very much from that of the empty carrier. Bolis *et al.* were studying exit experiments, and their explanation attributes the change in activation energy to a change in p, as defined in this paper (eqns. (1)–(3)), from a value considerably greater than one to a value considerably less than one over the experimental temperature range.

In this paper, the comparison of entry, exchange and exit experiments allows these suggestions to be tested for the leucine carrier system, and neither appears to account for the data. Two possibilities remain, both of which introduce an interesting new element into the carrier theory of transport processes. Either the equilibrium distribution of the carrier across the membrane must be asymmetrical and change very dramatically with changes in temperature, or the activation energy of *individual* carrier re-orientation steps must change very sharply with temperature. These possibilities are not mutually exclusive, and both may contribute to the observed activation energy changes. Both possibilities contain interesting implications for molecular mechanisms of carrier transport.

A further feature of interest is the different behaviour shown by the loaded and empty carriers in the leucine transport system. The ratio of re-orientation rate constants for the loaded to the empty carrier, p, increases sharply at low temperatures. This can only arise because the change in the activation energies of the mean re-orientation rate constants differ considerably in certain temperature ranges. Ultimately, the mechanism which accounts for these changes must also allow for differences between the behaviour of the loaded and empty carriers.

This ability of the membrane to discriminate between the loaded and empty carriers at low temperatures may have interesting physiological implications. The effect of these changes is to alter the relative efficiency of the carrier system for exchange and net transport. There is no evidence that the molecular changes occurring in the human erythrocyte membrane at low temperatures are related to the normal physiological control of the balance between exchange and net transport. However, these changes certainly represent one way in which this control can be effected, and are worthy of investigation.

All the experimental data indicate that some change in molecular

mechanism for leucine transport occurs between 10 and 20° C. This could be in a change of rate-determining step caused by large energy differences between the inward and outward orientated carriers, or it could lie with changes within the membrane itself, possibly involving structural changes in the lipid components. Further experiments are required to clarify and distinguish between these possibilities.

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